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Cellular regulation of dinoflagellate bioluminescence: Characterizing mechanosensitive ion channels in the signaling pathway

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The Thesis of Kelly Jin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Cellular regulation of dinoflagellate bioluminescence: Characterizing mechanosensitive ion channels in the signaling pathway

by

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Dinoflagellate bioluminescence represents a dramatic response to mechanical stress found in nature. The cellular mechanisms that govern this pathway, however, are not completely understood.

The objective of this thesis is to build and expand from previous studies to explore the mechanosensitive properties of dinoflagellate bioluminescence. Chapter I tests the hypothesis that the signaling pathway involves a stretch-activated component. Chapter I uses two separate, measurable types of bioluminescence in the dinoflagellate *Lingulodinium polyedrum* – mechanically-stimulated and spontaneous – as reporter

systems for pharmacological experiments that target specific areas of the pathway. Inhibition of both types of bioluminescence by the non-specific stretch-activated channel inhibitor gadolinium III and activation of bioluminescence by the membrane fluidizier benzyl alcohol suggest that a stretch-activated ion channel, in addition to a voltage-sensitive element, is involved in regulating dinoflagellate bioluminescence. Chapter II seeks to further characterize this stretch-activated ion channel, starting with a bioinformatic search of any existing dinoflagellates expressed sequence tags (ESTs) that represent mechanosensitive ion channels. This search resulted in partial sequences of a piezo-like mechanosensitive protein from *Karenia brevis* and a partial TRP-like channel from *Symbiodinium* sp. Furthermore, inhibition of bioluminescence and hypoosmotic cell-swelling by a TRP channel inhibitor, and activation of bioluminescence by a TRP channel activator, suggesting a role for TRP-like channels in the dinoflagellate bioluminescence signaling pathway. Based on these and previous studies, there are similar aspects of mechanosensory signaling seen between dinoflagellate bioluminescence and higher level eukaryotic mechanosensitive pathways, suggesting some level of conservation of mechanosensing proteins through eukaryotic evolution.

Chapter I:

Pharmacological evidence for the role of stretch-activated ion channels in the dinoflagellate bioluminescence signaling pathway

Summary

Dinoflagellate bioluminescence serves as a whole-cell reporter of mechanical stress, which activates a signaling pathway that appears to involve the opening of voltage-sensitive ion channels and release of calcium from intracellular stores. However, little else is known about the initial signaling events that facilitate the transduction of mechanical stimuli into cellular signaling events. In the present study using the red tide dinoflagellate *Lingulodinium polyedrum* (Stein) Dodge, two forms of dinoflagellate bioluminescence – mechanically stimulated and spontaneous flashes – were used as reporter systems to pharmacological treatments that targeted various predicted signaling events at the plasma membrane level of the signaling pathway. Treatment with 50 μM nifedipine, a voltage-sensitivity inhibitor that inhibits mechanically-stimulated bioluminescence, did not inhibit spontaneous bioluminescence, while pretreatment with 200 μM Gadolinium III (Gd^{3+}), a non-specific stretch-sensitive channel inhibitor, resulted in strong inhibition of all forms of bioluminescence. Treatment with 1 mM benzyl alcohol, a membrane fluidizer that activates mechanosensitive ion channels, but does not affect voltage-sensitivity, was very effective in stimulating bioluminescence, suggesting the involvement of a mechanosensitive component in the signaling pathway. Stimulation

by benzyl alcohol was also inhibited by Gd^{3+}. These results suggest that a stretch-activated ion channel and a voltage sensitive element is involved in the bioluminescence mechanotransduction signaling pathway.

Introduction

Bioluminescence, the emission of light by an organism as a result of the oxidation of a luciferin substrate, is widespread through the living kingdom (Haddock *et al.*, 2010). Common sources of bioluminescence in coastal regions are dinoflagellates, flagellate protists that in some cases form red tides resulting in spectacular displays of bioluminescence (Harrison, 1976, Rohr *et al.*, 1998).

Dinoflagellate bioluminescence is regulated by a mechanosensitive signaling pathway that results in the oxidization of the light-emitting substrate luciferin. The luciferin substrate and pH-dependent luciferase enzyme (Hastings & Dunlap, 1986) reside in scintillons, vesicles assembled in the Golgi apparatus that project into the vacuole space (Fogel & Hastings, 1972, Johnson *et al.*, 1985, Nicolas *et al.*, 1991). In some species, including *Lingulodinium polyedrum*, the luciferin is bound by a pH-dependent luciferin-binding protein (Fogel & Hastings, 1971, Wilson & Hastings, 1998). Upstream signaling events trigger the depolarization of the vacuole membrane resulting in a propagating action potential (Eckert, 1965a, Widder & Case, 1981). This depolarization opens voltage-gated proton channels (Smith *et al.*, 2011) resulting in the flux of protons from the acidic vacuole into the scintillons (Nawata & Sibaoka, 1979). The resulting acidification of the vacuole activates the luciferase, dissociates luciferin from its binding protein, allowing for the oxidization of luciferin resulting in light production (Fogel & Hastings, 1972, Wilson & Hastings, 1998). The entire

mechanotransduction signaling pathway leading to light emission is extremely rapid, with a delay from stimulus to the beginning of light emission of only 15-20 ms (Eckert, 1965b, Eckert & Sibaoka, 1968, Widder & Case, 1981, Latz *et al.*, 2008).

Despite a thorough characterization of the final luciferase-governed reaction that produces light, the signaling events preceding this final step are poorly understood. Mechanical stimulation causes an increase of cytosolic calcium concentration that appears to occur mainly from release from intracellular stores (von Dassow & Latz, 2002). However, the mechanotransduction process that ultimately increases cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) is still unknown. There is evidence that an increase in plasma membrane fluidity (Hardeland, 1980, Mallipattu *et al.*, 2002) and activation of heterotrimeric GTP-binding proteins (G-proteins) (Chen *et al.*, 2007) play a role in mechanosensing, as in mammalian endothelial cells (Haidekker *et al.*, 2000, Gudi *et al.*, 1998). However, pharmacological evidence for the involvement of plasma membrane voltage-sensitive ion channels in the dinoflagellate bioluminescence signaling pathway (von Dassow, 2003) necessitates further study to elucidate the mechanism of mechanosensing in dinoflagellates.

The red-tide species *Lingulodinium polyedrum* (Lewis & Hallett, 1997) is a classic system for studying the signaling pathway of dinoflagellate bioluminescence. *L. polyedrum* produces two measurable forms of light that were used as the reporter systems of the present study. The first is mechanically-stimulated bioluminescence, which is a response to mechanical stress (Maldonado & Latz, 2007). The mechanosensitivity of dinoflagellates such as *L. polyedrum* is tuned such that their response threshold is not triggered by background ocean flows but is sensitive enough to be stimulated by predator

contact (Latz & Rohr, 1999, Latz *et al.*, 2004). A mechanically stimulated flash of *L. polyedrum* is approximately 100 ms in duration and contains 10^8 photons $cell^{-1}$ (Latz *et al.*, 1987, Latz & Lee, 1995). The second form of bioluminescence is released in the form of spontaneous flashes and glow (Krasnow *et al.*, 1980), which occur in the absence of any mechanical stimuli (Latz & Lee, 1995). In dinoflagellates, spontaneous bioluminescence is governed by a circadian rhythm and is composed of flashing and a persistent glow (Krasnow et al., 1980). Although the temporal pattern of flashing over time is random, the flashes occur at a predictable average rate of ~0.002 flashes min^{-1} $cell^{-1}$ for *L. polyedrum* (Krasnow et al., 1980, Latz & Lee, 1995). The present study employed a pharmacological approach to characterize components of the dinoflagellate bioluminescence signaling pathway using treatments involving spontaneous, mechanically-induced, and chemically-activated luminescence. Results provide evidence for the involvement of a stretch-activated ion channel and another voltage-sensitive component.

Methods

Cell culture and preparation

Cultures of *Lingulodinium polyedrum* (Stein) Dodge, 1989 strain CCMP1937 (formerly known as *Gonyaulax polyedra* Stein) were grown in f/2 medium in an environmental chamber maintained at 20.0 ± 0.5°C on a 12:12 h light-dark cycle. Cell concentration was estimated on each day of testing by replicate counts of subsamples of known volume under a dissecting microscope. Experiments were performed on cultures within the exponential phase of growth with cell concentrations within the range of 1,000-8,000 cells/ml.

Subsamples of culture were prepared at the end of the light phase when cells are mechanically inexcitable (Biggley *et al.*, 1969). Samples were placed in 7 ml glass scintillation vials. All samples were measured at 20.0±0.5°C, 4-7 h into the dark phase when maximal levels of bioluminescence are present (Biggley et al., 1969, Latz & Lee, 1995).

Experiments were typically set up using a 2 x 2 factorial design, with a pretreatment factor (inhibitor or control condition) and a treatment factor (activator or control condition). Each experiment consisted of four replicate samples for each combination of treatments (i.e., control pretreatment and control treatment, control pretreatment and activating treatment, inhibiting pretreatment and control treatment, and inhibiting pretreatment and activating treatment. Each experiment was replicated twice. All pretreatments were given 40 min prior to the treatment.

Media and chemicals

Filtered seawater (FSW) was prepared with GF/F filtered full-strength seawater collected from Scripps Pier at University of California, San Diego. Gadolinium (III) chloride (Gd^{3+}), Benzyl alcohol, BAPTA-AM, and Nifedipine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium Chloride (KCl) and Dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Stock solutions of Nifedipine and BAPTA-AM were prepared in DMSO and immediately diluted in FSW. All Gadolinium (III) chloride, Benzyl alcohol, and KCl solutions were prepared in FSW. All working solutions were made fresh daily. Concentrations represent the final concentration after addition of the chemical.

Bioluminescence measurements

Bioluminescence was measured in a 15 cm diameter light-integrating chamber (Labsphere Inc., North Sutton, NH, USA) using a photon-counting photomultiplier detector (Electron Tubes model P30232, ET Enterprises Ltd., Uxbridge, UK) fitted with a Uniblitz electronic shutter (Vincent Associates, Falmouth, MA, USA). A baffle blocked direct view of the sample vial by the detector, assuring an integrated light measurement due to highly reflective white inner surface of the chamber. In some cases the light signal was attenuated to 10% transmission with a 1.0 neutral density filter (Kodak Wratten #96, Eastman Kodak Co., Rochester, NY, USA) to avoid saturation of the detector. Light readings were acquired based on 10 ms integrations. Appropriate chemical pretreatments were administered to each sample 40 min before testing. Chemical pretreatments, including controls, consisted of 0.1 ml volumes that were manually administered slowly to minimize prestimulation of bioluminescence. Spontaneous and injection-treated bioluminescence were recorded for 5 min. Stirring-induced bioluminescence was recorded for 1 min. All experiments were carried out in a dim light environment to avoid photoinhibition of bioluminescence (Hamman & Seliger, 1982).

For measurements of spontaneous bioluminescence, samples were gently fixed in a holder and placed in the light-integrating chamber (Fig. 1A). Spontaneous flashes and glow were measured with the photomultiplier detector over 5 min without disturbance. Chemical treatments, including KCl and Benzyl alcohol, and the appropriate controls, consisted of 0.1 ml volumes that were administered using a syringe pump (Harvard Apparatus, Holliston, MA, USA) at an infusion rate of 1 ml min^{-1} (Fig. 1B) to minimize mechanical stimulation by the introduced liquid. Bioluminescence was then measured for

5 min. Bioluminescence mechanically stimulated by the addition of the fluid (usually the first 6 s) was not included in the final data analysis.

For mechanical stimulation of bioluminescence, samples were gently placed in a custom-designed holder that included a motorized stirrer (Fig. 1C). Stirring-induced bioluminescence was then measured over 1 min. Stirring occurred at a constant rate throughout all experiments.

Assay for total luminescent capacity

Immediately following bioluminescence measurements as previously described, residual light capacity of cells was assayed via cell acidification. This causes the dissociation of luciferin from its binding protein and activation of luciferase, which is inactive at physiological pH, to oxidize any remaining luciferin. A Sirius luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) was used to simultaneously inject 250µl of 1 M acetic acid and measure light emission over 60 s, with the results expressed in relative light units (RLU s^{-1} $cell^{-1}$). The sum of mechanical or chemical stimulation plus the luminometer acidification was the total stimulated luminescence (TSL), representing the luminescent capacity of the cells. TSL was used to assess the toxicity of pharmacological treatments. Unless otherwise stated, there was no significant effect of the treatments on TSL based on statistical analysis.

Statistical analysis

Unless otherwise stated, all values are expressed as mean ± standard deviation. Statistical analyses were performed using JMP 9 software (SAS Institute Inc., Cary, NC, USA). Simple comparisons used Student's *t*-test. Analysis of variance for the 2 x 2 factorial experimental design was done with a mixed model using the REML (REsidual

Maximum Likelihood) method, with the pretreatment and treatment conditions as fixed effects and experiment date as a random effect. Statistical significance was based on $P < 0.05$.

Results

Nifedipine affects mechanically-stimulated and KCl-stimulated bioluminescence but not spontaneous bioluminescence

Nifedipine, a dihydropyridine calcium channel blocker (Table 1.1) that is used as an inhibitor of voltage-gated cation channels, inhibits flow-stimulated and K^+-induced bioluminescence of *L. polyedrum* (von Dassow, 2003). These results were replicated in this study with the enhanced protocol for measuring bioluminescence. Pretreatment by 50 μM nifedipine inhibited mechanical stimulation of bioluminescence by stirring compared to the 0.1% DMSO control pretreatment (Fig. 2A). Overall, there was an integrated luminescence of 495.4 ± 103.3 photons s^{-1} $cell^{-1}$ for the 0.1% DMSO pretreatment, while the 50 μM nifedipine pretreatment condition had an integrated luminescence of 192.5 ± 171.5 photons s^{-1} $cell^{-1}$ (Fig. 2B). This 61% inhibition of stirring-induced bioluminescence represented a significant effect of nifedipine pretreatment on mechanical stimulation by stirring (t-test, $t_{14} = 16.02$, $p = 0.0018$) and was similar to the 67% inhibition of shear-stimulated luminescence obtained by von Dassow (2003).

Treatment with 30 mM KCl stimulated bioluminescence (Fig. 2C-D), confirming earlier work (von Dassow & Latz, 2002, von Dassow, 2003). Overall the integrated luminescence for KCl treatment was 29.1 ± 15.6 photons s^{-1} $cell^{-1}$, compared to 1.3 ± 0.7 photons s^{-1} $cell^{-1}$ for the FSW control treatment (Fig. 2E), indicating a significant effect of the KCl treatment on bioluminescence (ANOVA, $F_{1,44} = 71.32$, $p < 0.0001$).

Pretreatment with 50 μM nifedipine inhibited K^+ stimulation of bioluminescence by 87% compared to the DMSO control pretreatment, based on an integrated luminescence of 17.7 ± 6.1 photons s^{-1} $cell^{-1}$ for the nifedipine pretreatment and 29.1 ± 15.6 photons s^{-1} $cell^{-1}$ for the DMSO pretreatment. The interaction between nifedipine pretreatment and KCl treatment was significant (ANOVA, $F_{1,42} = 5.51$, $p = 0.0237$). There was no significant effect of 50 μM nifedipine pretreatment on the control FSW treatment (ANOVA, $F_{1,44} = 5.91$, $p = 0.9999$); integrated luminescence was 1.1 ± 0.4 photons s^{-1} $cell^{-1}$ for the DMSO control pretreatment and 1.3 ± 0.7 photons s^{-1} $cell^{-1}$ for the Nifedipine pretreatment. There were no statistical differences among TSL values for all conditions (ANOVA, $F_{1,40} = 0.53$, $p = 0.4723$), indicating that Nifedipine did not have a toxic effect on the cells (data not shown).

Nifedipine pretreatment did not have a significant effect on spontaneous bioluminescence. The integrated luminescence of the 0.1% DMSO control pretreatment was 0.87 ± 0.46 photons s^{-1} $cell^{-1}$, while that of the 50 μM nifedipine pretreatment was 0.79 ± 0.321.7 photons s^{-1} $cell^{-1}$ (*t*-test, $t_{14} = 0.38$, $p = 0.6470$) (Fig. 3A). Integrated spontaneous luminescence consists of the rate of spontaneous flashing and flash intensity. The spontaneous flash rate of the 0.1% DMSO control was 1.2 ± 0.6 x 10^{-3} flashes $cell^{-1}$ min^{-1}, and the spontaneous flash rate for nifedipine-pretreated cells was 1.2 ± 1.2 x 10^{-3} flashes $cell^{-1}$ min^{-1}, suggesting that nifedipine pretreatment had no significant effect on spontaneous flash rate (*t*-test, $t_{14} = 0.02$, $p = 0.4910$) compared to DMSO controls (Fig. 3B). The average spontaneous flash intensity was 2.9 ± 0.3 x 10^5 photons s^{-1} for the 0.1% DMSO control and 2.7 ± 0.8 x 10^5 photons s^{-1} for nifedipine-pretreated cells, indicating that nifedipine pretreatment also had no significant effect on average flash intensity (*t*-

test, t_{14} = 0.47, p=0.6782). TSL values for both the DMSO control pretreatment and nifedipine pretreatment were not significantly different (*t*-test, t_{14} = 0.32, p = 0.6253), indicating that nifedipine did not have a toxic effect on cells (data not shown).
The lack of inhibition of spontaneous bioluminescence by nifedipine suggests that the origin of spontaneous flashes in *L. polyedrum* is not from a voltage-sensitive source.

BAPTA-AM significantly inhibits spontaneous flash rate

BAPTA-AM inhibits mechanically-stimulated and KCl-stimulated bioluminescence in *L. polyedrum* by chelating free cytosolic Ca^{2+} whose main origin is release from intracellular stores (von Dassow & Latz, 2002). The effect of BAPTA-AM on spontaneous bioluminescence was tested to determine if the spontaneous activity had a plasma membrane or cytoplasmic origin.

BAPTA-AM pretreatment strongly inhibited spontaneous bioluminescence (Fig. 4A, B). Integrated spontaneous bioluminescence over 300 s duration of the 0.04% DMSO pretreatment was 2.2 ± 0.6 photons s^{-1} $cell^{-1}$, while that of the 2 μM BAPTA-AM pretreatment was 0.7 ± 0.1 photons s^{-1} $cell^{-1}$; this 69% decrease represented a significant difference (*t*-test, t_8 = 5.42, p = 0.0003) (Fig. 4C). The decrease in integrated spontaneous luminescence was primarily due to a 95% inhibition of spontaneous flashing, from a flash rate of 9.5 ± 2.5 x 10^{-3} flashes $cell^{-1}$ min^{-1} for the DMSO control pretreatment to 4.9 ± 5.5 x 10^{-4} flashes $cell^{-1}$ min^{-1} for the BAPTA-AM pretreatment, representing a significant difference (*t*-test, t_8 = 7.78, p < 0.0001) (Fig. 4D). The flash intensity of those few spontaneous flashes that were produced for the BAPTA-AM pretreatment was 2.3 x 10^5 photons s^{-1}, compared to 3.8 x 10^5 photons s^{-1} for the DMSO control pretreatment; this 59% decrease represented a significant difference (*t*-test, t_8 = 2.41, p = 0.0213) (Fig. 4E).

TSL for the BAPTA-AM pretreated cells was 1.1x higher and significantly different (t-test, $t_8 = 3.10$, p=0.0146) from that for the DMSO-pretreated control, indicating that BAPTA-AM-pretreated cells contained more residual bioluminescence as a result of inhibition and that BAPTA-AM did not have a toxic effect on cells (data not shown). The dramatic inhibition of BAPTA-AM on spontaneous bioluminescence further suggests that the origin of spontaneous flashes is upstream from the increase in $[Ca^{2+}]_{cyt}$ that occurs during activation of the signaling pathway. The lack of inhibition of the rate of spontaneous flashing by nifedipine suggests that there is another upstream signaling component involved in bioluminescence regulation in *L. polyedrum*.

Gd^{3+} strongly inhibits stirring-induced and spontaneous bioluminescence

The inhibition of mechanically stimulated bioluminescence in *L. polyedrum* by gadolinium III (Gd^{3+}) (von Dassow, 2003), a non-specific inhibitor of stretch-activated channels (Yang & Sachs, 1989, Berrier *et al.*, 1992, Hajduczok *et al.*, 1994), suggests that the signaling pathway possesses a stretch-activated component.

Gd^{3+} strongly inhibited stirring-induced bioluminescence in *L. polyedrum*, confirming earlier work (von Dassow, 2003). Stirring-stimulated integrated luminescence over 60 s of the FSW control pretreatment was 472.6 ± 37.0 photons s^{-1} $cell^{-1}$, while that of the Gd^{3+} pretreatment was 19.8 ± 6.4 photons s^{-1} $cell^{-1}$; this 96% decrease represented a significant difference (*t*-test, $t_{14} = 37.15$, $p < 0.0001$) (Fig. 5A, B). TSL for the Gd^{3+}-pretreated cells was significantly higher than TSL for the control, suggesting that Gd^{3+}-pretreated cells contained more residual bioluminescence as a result of inhibition (*t*-test, t_{14} 2.64, p = 0.0098), indicating that Gd^{3+} did not have a toxic effect on cells (data not shown).

Gd^{3+} also inhibited spontaneous bioluminescence. The integrated spontaneous luminescence over 300 s of the FSW control pretreatment was $5.5 \pm 0.5 \times 10^{-1}$ photons s^{-1} $cell^{-1}$, while that of the Gd^{3+} pretreatment was $3.3 \pm 0.6 \times 10^{-1}$ photons s^{-1} $cell^{-1}$; this 69% decrease represented a significant difference (*t*-test, $t_{14} = 7.81$, $p < 0.0001$) (Fig. 5C). This inhibition was due to a decrease in spontaneous flash rate by Gd^{3+} (Fig. 5D). The spontaneous flash rate of the FSW control pretreatment was $7.3 \pm 2.0 \times 10^{-4}$ flashes $cell^{-1}$ min^{-1}, while that of the Gd^{3+} pretreatment was $2.3 \pm 1.1 \times 10^{-4}$ flashes $cell^{-1}$ min^{-1}; this 69% decrease represented a significant decrease (*t*-test, $t_{14} = 6.50$, $p < 0.0001$). There was no significant effect of Gd^{3+} on spontaneous flash intensity (*t*-test, $t_{14} = 0.12$, $p = 0.9043$) (Fig. 5E).

The strong inhibition by Gd^{3+} on spontaneous as well as mechanically stimulated bioluminescence provides positive evidence for stretch-activated channels involved in the bioluminescence signaling mechanism of *L. polyedrum*.

<u>Gd^{3+} but not nifedipine inhibits benzyl alcohol activation of bioluminescence</u>

To further explore the effect of membrane fluidity on stretch-sensitive channels, cells were treated with benzyl alcohol, a membrane fluidizer (Zhang *et al.*, 2011, Blixt *et al.*, 1993) and activator of G proteins (Chachisvilis *et al.*, 2006).

Addition of benzyl alcohol caused immediate and prolonged chemical stimulation of bioluminescence (Fig. 6A,B). The integrated luminescence over 300 s for the FSW control treatment was 0.9 ± 0.2 photons s^{-1} $cell^{-1}$, while that for the 1 mM benzyl alcohol treatment was 15.1 ± 10.7 photons s^{-1} $cell^{-1}$ (Fig. 6C); this 17x increase represented a highly significant difference (*t*-test, $t_{14} = 3.76$, $p = 0.0021$).

If benzyl alcohol is acting via a non-voltage gated mechanism involving a stretch-

activated ion channel, then Gd^{3+} is expected to inhibit benzyl alcohol stimulation. As expected, pretreatment with 200 μM Gd^{3+} inhibited bioluminescence activation by 1 mM benzyl alcohol (Fig. 7A,B). Overall the Gd^{3+} pretreatment inhibited benzyl alcohol-stimulated bioluminescence by 95% compared to the FSW control pretreatment, with an integrated luminescence over 300 s of only 0.7 ± 0.6 photons s^{-1} $cell^{-1}$ for the Gd^{3+} pretreatment compared to 15.1 ± 10.7 photons s^{-1} $cell^{-1}$ for the FSW control (Fig. 7C); this represented a significant interaction (ANOVA, $F_{1,27} = 15.01$, $p = 0.0006$). As expected, Gd^{3+} pretreatment significantly decreased bioluminescence by 20% compared to the FSW control treatment (ANOVA, $F_{1,27} = 15.81$, $p = 0.0005$) (Fig. 7C). TSL for the Gd^{3+} -pretreated cells was 1.1x higher and significantly different than TSL for the control pretreatment (ANOVA, $F_{1,25} = 20.87$, $p<0.0001$), indicating that Gd^{3+}-pretreated cells contained more residual bioluminescence as a result of inhibition and indicating that Gd^{3+} did not have a toxic effect on cells (data not shown).

If bioluminescence is activated via a non-voltage gated mechanism involving an ion channel, then it is expected that nifedipine would not inhibit benzyl alcohol stimulation. There was no significant interaction of 50 μM nifedipine pretreatment with benzyl alcohol-stimulated bioluminescence (ANOVA, $F_{1,27} = 0.14$, $p = 0.7100$) (Fig. 7D); the integrated luminescence over 300 s after 1 mM benzyl alcohol addition was 11.1 ± 9.9 photons s^{-1} $cell^{-1}$ for the nifedipine pretreatment and 9.3 ± 3.3 photons s^{-1} $cell^{-1}$ for the DMSO control pretreatment. TSL for the nifedipine and DMSO pretreatments were not significantly different (ANOVA, $F_{1,27} = 0.32$, $p = 0.5756$), indicating that nifedipine did not have a toxic effect on cells (data not shown).

Discussion

The present results support our hypothesis that the mechanosensitive dinoflagellate bioluminescence signaling pathway involves non-voltage-gated elements, while spontaneous bioluminescence involves both or either electrical and mechanical signaling events. This conclusion is based on the dramatic inhibition of both mechanically-induced and spontaneous bioluminescence by Gd^{3+}, a non-specific inhibitor of stretch-activated channels; stimulation of bioluminescence by benzyl alcohol, a membrane fluidizer that is known to activate G proteins and stretch-activated channels; inhibition of benzyl alcohol stimulation by Gd^{3+} pretreatment; and lack of inhibition of benzyl alcohol stimulation by nifedipine, a blocker of voltage-gated cation channels. Dinoflagellate bioluminescence has both deterministic and stochastic components. Mechanically-stimulated bioluminescence is a result of mechanical stress that triggers the initiation of the signaling pathway. On the other hand, some dinoflagellate species such as *L. polyedrum* exhibit spontaneous bioluminescence that occurs in the absence of mechanical stress, and is composed of flash and glow components that are governed by independent circadian rhythms (Hastings & Sweeney, 1958, Krasnow et al., 1980, Heyde *et al.*, 1992). The peak in spontaneous glowing at the end of the dark phase may be due to the breakdown of scintillons (Fritz *et al.*, 1990), but spontaneous glowing occurs at low levels throughout the dark phase and appears to be due to leakage of protons from the vacuole (Behrmann & Hardeland, 1995) based on pharmacological evidence involving stimulation of glowing by indoleamines and monoamine inhibitors and inhibition by V-type ATPase inhibitors (Balzer & Hardeland, 1991, Burkhardt & Hardeland, 1996, Burkhardt *et al.*, 1997).

We propose that spontaneous flashing occurs through a completely different mechanism involving one or more noisy components of the signaling pathway located in the plasma membrane. Many types of animal cells exhibit fluctuations in plasma membrane potential due to the opening and closing of ion channels, leading to spontaneous activity that is important in oscillatory rhythms, circadian rhythms, and complex brain function (Ermentrout *et al.*, 2008, Lundkvist & Block, 2005, Moss *et al.*, 2004). In unicellular eukaryotes these fluctuations can be associated with behavioral changes. For example, the ciliated protist *Paramecium* exhibits spontaneous changes in swimming direction associated with fluctuations in membrane potential that cause a reversal in ciliary beating (Machemer, 1989, Naitoh, 1974). The membrane potential typically fluctuates 1-3 mV (Majima, 1980, Moolenaar *et al.*, 1976); a sufficient fluctuation causes a spike-like depolarization that that opens voltage-gated Ca^{2+} channels in the ciliary membrane (Dunlap, 1977, Ogura & Takahashi, 1976) and causes a release of Ca^{2+} from intracellular stores (Plattner *et al.*, 2006) that increases $[Ca^{2+}]_{cyt}$ to open more Ca^{2+} channels (Nakaoka *et al.*, 2009). Other ciliates show similar relationships between fluctuations in cell membrane potential and swimming behavior (Berg & Sand, 1994, Lueken *et al.*, 1996, Rudberg & Sand, 2000). So fluctuations in membrane potential lead to so-called spontaneous changes in organism behavior.

Using spontaneous flashing as a reporter system for various inhibitory treatments allowed us to determine the relative location of this source of noise in the dinoflagellate signaling pathway. Inhibition of spontaneous flashing by BAPTA-AM pretreatment indicates that the origin of noise involves a component of the signaling pathway upstream of the increase in $[Ca^{2+}]_{cyt}$. The inhibition of spontaneous bioluminescence by Gd^{3+}

suggests that the source of noise is either a stretch-activated channel or a component upstream of the channel.

Although voltage-sensitive channels have been previously proposed to be involved in the mechanosensitive dinoflagellate bioluminescence signaling pathway (von Dassow, 2003), the lack of inhibition of spontaneous flashes by nifedipine suggests an alternate spontaneous signaling component. Evidence from this study suggests this alternate component is a stretch-activated channel. The strong inhibition by Gd^{3+} on benzyl alcohol-induced bioluminescence, and the lack of inhibition by nifedipine, provided further evidence that benzyl alcohol is acting through stretch activation rather than voltage activation. These results imply that Gd^{3+} and nifedipine target different sensitivities of proteins, and therefore suggests stronger stretch activation rather than voltage activation.

Although mechanically-stimulated bioluminescence is inhibited by both Gd^{3+} and nifedipine (von Dassow, 2003), nifedipine pretreatment resulted in a much weaker inhibition compared to Gd^{3+} pretreatment. This suggests that the mechanosensitive pathway involves a stretch-activated component that possesses some voltage sensitivity. Mechanosensitive channels can also be sensitive to changes in voltage (Laitko & Morris, 2004, Lin *et al.*, 2007), while many voltage-gated channels can also change their structural conformation as a result of membrane stress in the same way as can mechanosensitive channels (Laitko *et al.*, 2006, Morris & Juranka, 2007). Although the pharmacological approach taken by this study is useful in characterizing potential components of the signaling system, identification of the signaling proteins requires a molecular approach. Unfortunately, the dinoflagellate genome possesses unique

characteristics that have to date challenged genome sequencing attempts. It consists of approximately 215,000 megabase pairs – close to 100x that of human cells, most likely due to genome replication, and resides in hundreds of condensed chromosomes (Dodge, 1985, Rizzo, 1991). However, dinoflagellate EST databases and soon-to-be-available dinoflagellate transcriptomes are tools for identifying and sequencing the genes of potential signaling molecules (Hackett *et al.*, 2005).

The evidence for a stretch-activated channel involved in bioluminescence signaling is perhaps not surprising due to the mechanosensitive properties of bioluminescence in *L. polyedrum*. However, this finding plays a significant role in further understanding the properties of mechanosensing. Components of the dinoflagellate bioluminescence signaling pathway appear to be conserved in mechanosensitive cells of higher organisms. In dinoflagellates there is evidence for stress-induced changes in membrane fluidity (Mallipattu et al., 2002), stimulation of bioluminescence by membrane fluidization (Hardeland, 1980), a role of G-proteins in mechanotransduction (Chen et al., 2007), as well an increase in $[Ca^{2+}]_{cyt}$ (von Dassow & Latz, 2002). In mammalian endothelial cells, shear stress rapidly activates G-proteins via an increase in membrane fluidity (Chachisvilis et al., 2006, Gudi et al., 1998), leading to an increase in $[Ca^{2+}]_{cyt}$ (Ando *et al.*, 1994), although this response to shear stress occurs at a longer time scale of about 5 s (Gudi *et al.*, 2003) while the response latency for dinoflagellate bioluminescence is only 15-20 ms (Eckert, 1965a, Widder & Case, 1981, Latz et al., 2008). Evidence from this study suggests, however, the presence of an ion channel, possibly in addition to G protein activation, that is triggered by mechanical stimulation.

The following is a hypothesized sequence of upstream signaling events that links

our current study with previously proposed signaling events. Mechanical stress causes an increase in fluidity of the plasma membrane, which results in G protein activation. This process can be mimicked by increasing plasma membrane fluidity via benzyl alcohol treatment. The type of G protein is unknown, but a G_q protein is hypothesized based on the known involvement of intracellular calcium (von Dassow & Latz, 2002) and the involvement of G_q proteins in mechanotransduction by mammalian endothelial cells. The downstream effects of G_q protein activation include the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 then acts as a secondary messenger that opens IP_3-gated calcium channels that regulate intracellular calcium release from stores (Ando et al., 1994). In both mammalian arterial and cardiac muscle cells, DAG has been shown to open plasma membrane ion channels that facilitate the transduction of mechanical stimuli into a downstream, cellular response (Sharif-Naeini *et al.*, 2010). Although it is yet unclear if these ion channels can be directly activated by mechanical stress, they are known to be inhibited by Gd^{3+} (Wolfle *et al.*, 2010, Olsen *et al.*, 2011, Ryskamp *et al.*, 2011). Based on the current results, we propose that the dinoflagellate bioluminescence signaling pathway involves stress-activated G proteins that open Gd^{3+}-sensitive ion channels to allow Ca^{2+} influx that depolarizes the cell, and that spontaneous flashing occurs through the stochastic activation of the G proteins, leakage of Ca^{2+} through the Gd^{3+}-sensitive ion channels, or both.

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Table 1.1. Pharmacological treatments used in this study.

Chemical	Action	Known/predicted effect on bioluminescence
Nifedipine	L-type Ca^{2+} channel blocker	Inhibits
KCl	Depolarizes plasma membrane	Activates
BAPTA-AM	Chelates free cytosolic Ca^{2+}	Inhibits
Gadolinium III chloride (Gd^{3+})	Inhibits stretch-sensitive channels	Inhibits
Benzyl alcohol	Increases membrane fluidity	Activates

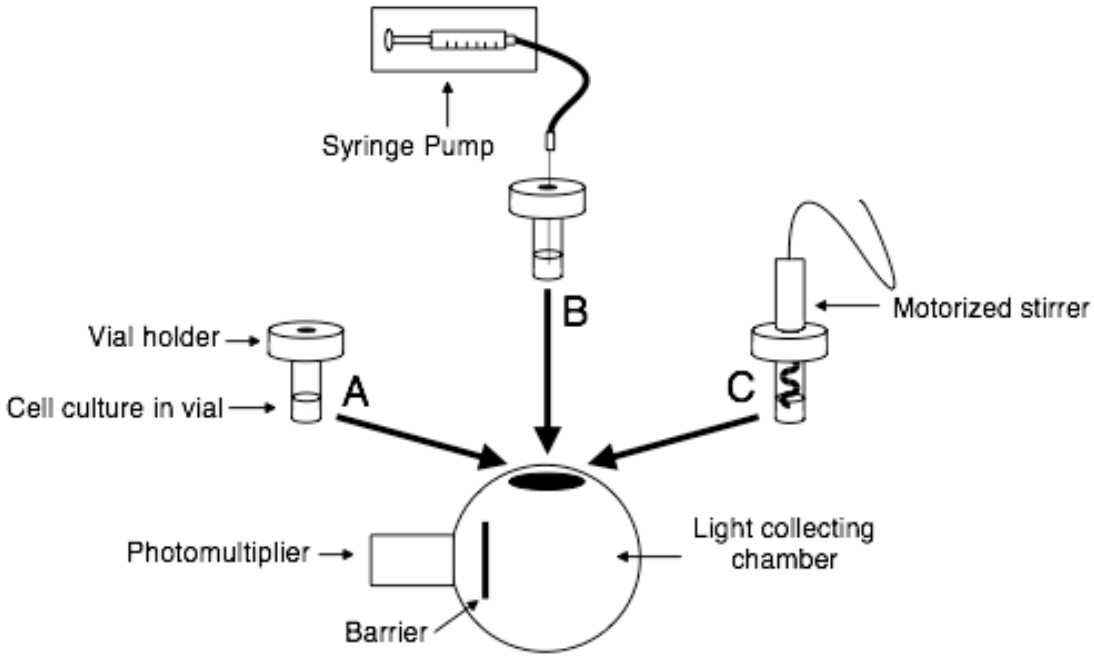

Figure 1.1. Schematic description of bioluminescence measurements. Bioluminescence was measured in a light-collecting chamber by a photon-counting photomultiplier. A barrier placed in front of the photomultiplier allowed for an integrated view of light produced in the chamber. (A) For measurements of spontaneous bioluminescence, the sample vial was attached to a holder that was mounted to the top of the chamber. (B) For chemically-stimulated bioluminescence measurements, a syringe needle connected by tubing to an automated syringe pump was inserted into the vial holder to allow for a controlled rate of injection (1 ml min^{-1}). (C) For stirring-induced bioluminescence measurements, a motorized stirrer mixed the contents of the vial to produce mechanical stimulation.

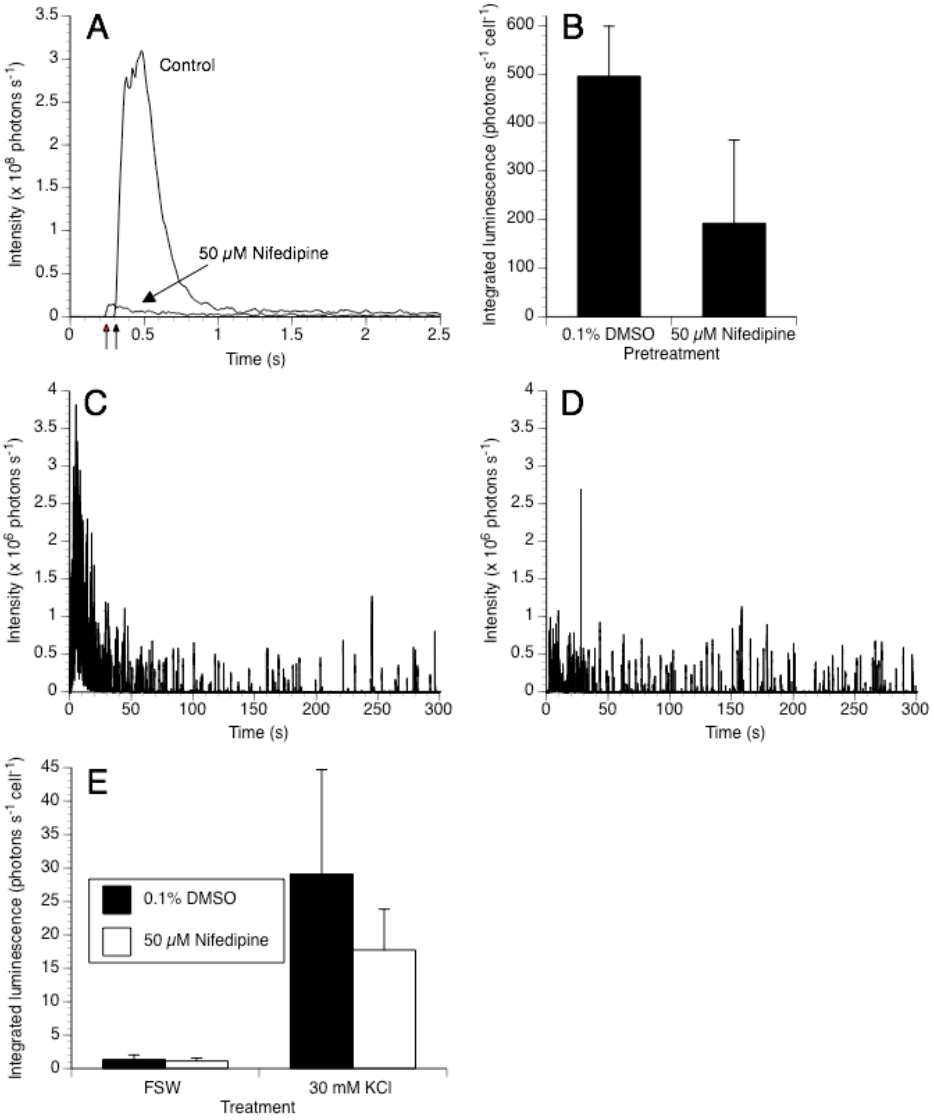

Figure 1.2. Effect of nifedipine pretreatment on stirring and KCl-induced bioluminescence. (A) Representative record of initial bioluminescence stimulated by stirring in ~3300 cells pretreated with 0.1% DMSO as a control or 50 µM nifedipine. Arrows below the x-axis indicate the initiation of stirring. (B) Summary of the effect of 50 µM nifedipine on 60 s integrated luminescence stimulated by stirring. Values represent the average ± SD of 10-15 replicates. Stirring-induced bioluminescence by nifedipine-pretreated cells was 61% lower and significantly different ($p = 0.0018$) than the DMSO control. (C) Representative record of 30 mM KCl-stimulated bioluminescence in ~1300 cells pretreated with 0.1% DMSO as the control. (D) Similar record for KCl treatment following 50 µM nifedipine pretreatment. (E) Summary of the effect of 30 mM KCl treatment on 300 s integrated luminescence in cells pretreated with 0.1% DMSO (closed bars) or 50 µM nifediepine (open bars). Values represent the average ± SD of 8 replicates. KCl-stimulated bioluminescence by nifedipine-pretreated cells was 39% lower and significantly different ($p = 0.0083$) than the DMSO control.

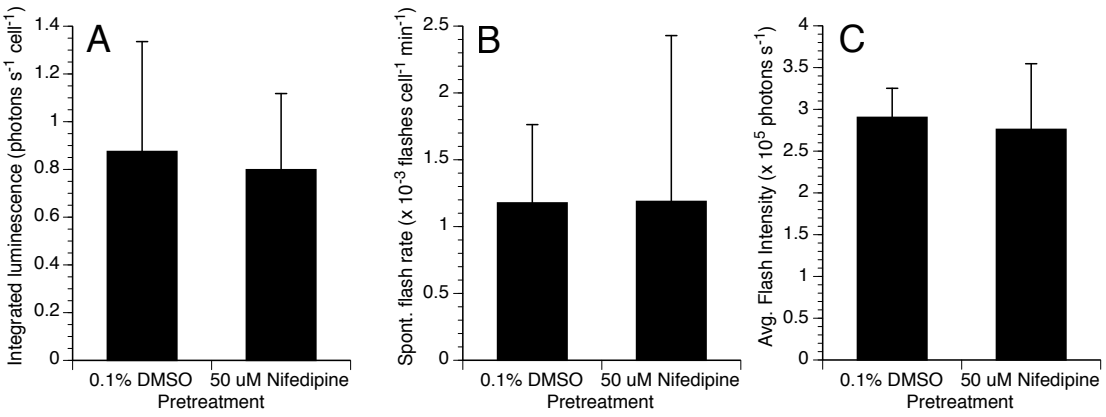

Figure 1.3. Effect of nifedipine pretreatment on spontaneous bioluminescence. All values represent average ± SD of 8 replicates for records of 300 s duration. (A) Summary of the effect of 50 μM nifedipine pretreatment on integrated luminescence. There was no significant effect of nifedipine pretreatment on integrated bioluminescence, compared to the DMSO control ($p = 0.7061$). (B) Effect on spontaneous flash rate. There was no significant effect of nifedipine pretreatment on spontaneous flash rate compared to the DMSO control ($p = 0.9820$). (C) Effect on average flash intensity. There was no significant effect of nifedipine pretreatment on spontaneous flash rate compared to the DMSO control ($p = 0.6436$).

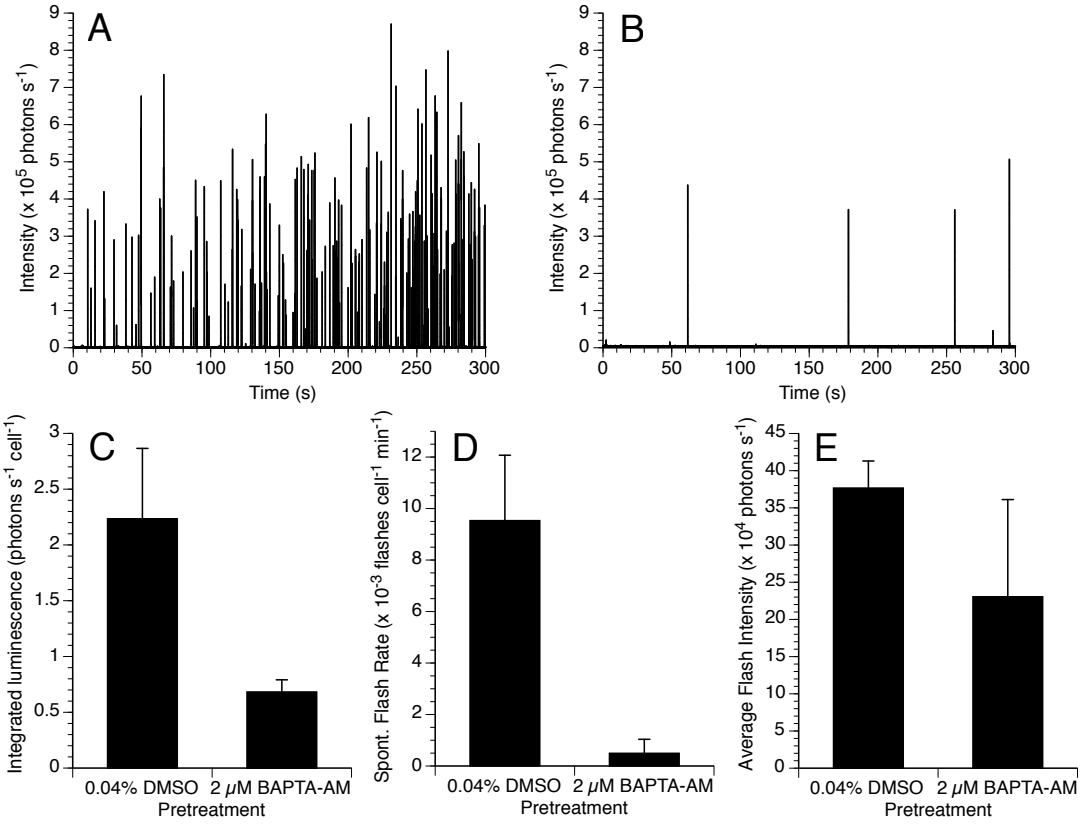

Figure 1.4. Effect of BAPTA-AM pretreatment on spontaneous bioluminescence. Representative records of spontaneous bioluminescence in ~6000 cells pretreated with (A) 0.04% DMSO as the control, or (B) 2 μM BAPTA-AM. (C) Effect of BAPTA-AM on integrated luminescence for spontaneous bioluminescence measured over 300 s. Integrated luminescence for BAPTA-AM pretreatment was 69% lower and significantly different ($p < 0.001$) than the DMSO control. (D) Effect of BAPTA-AM on spontaneous flash rate. The spontaneous flash rate for BAPTA-AM treated cells was 95% lower and significantly different ($p<0.0001$) than the DMSO control. (E) Effect of BAPTA-AM on average flash intensity. Spontaneous flash intensity for BAPTA-AM pretreatment was 59% lower and significantly different ($p=0.043$) than the DMSO control. Values in (C-E) represent the average ± SD of 5 replicates.

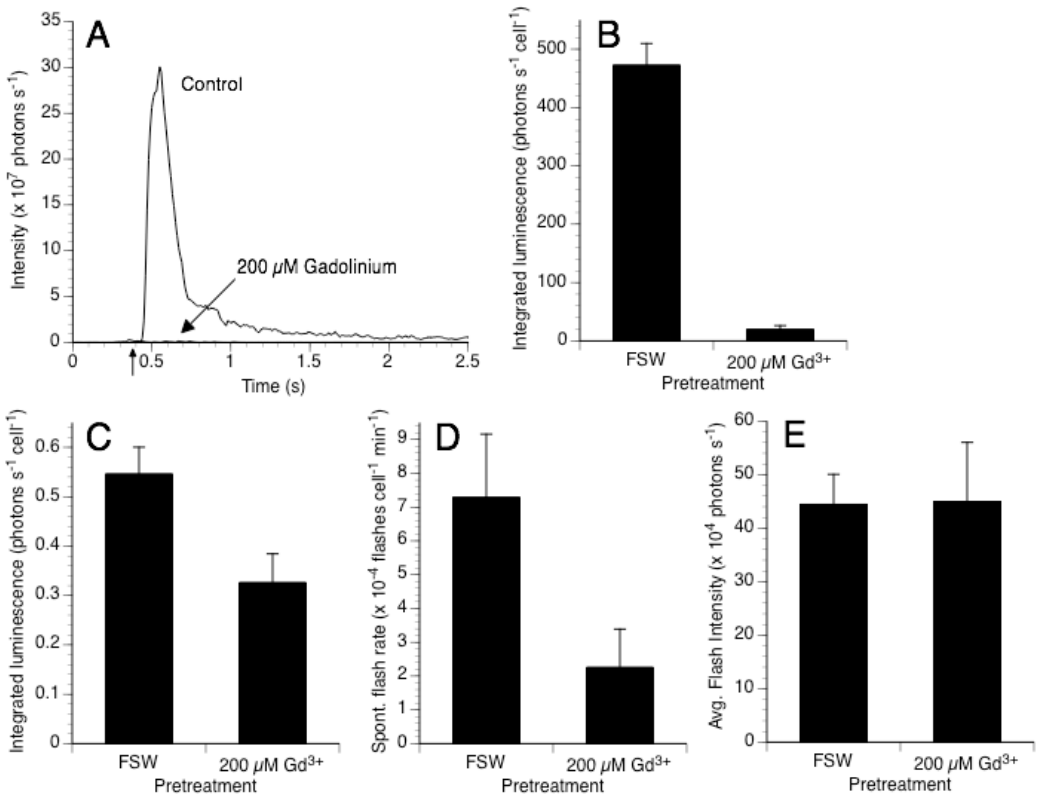

Figure 1.5. Effect of Gd^{3+} pretreatment on mechanically stimulated and spontaneous bioluminescence. (A) Representative records of initial bioluminescence stimulated by stirring in ~3000 cells pretreated with FSW as the control or 200 µM Gd^{3+}. Arrow below the x-axis indicates the initiation of stirring. (B) Summary of the effect of Gd^{3+} pretreatment on integrated stirring-induced luminescence over 60 s. Integrated bioluminescence for the Gd^{3+} pretreatment was 96% lower and significantly different ($p<0.0001$) than the FSW control. (C) Summary of the effect of Gd^{3+} on integrated spontaneous bioluminescence measured over 300 s. Integrated spontaneous bioluminescence for Gd^{3+} pretreatment was 69% lower and significantly different ($p<0.0001$) than the FSW control. (D) Summary of the effect of Gd^{3+} pretreatment on spontaneous flash rate. The spontaneous flash rate for Gd^{3+} pretreatment was 69% lower and significantly different ($p<0.0001$) than the FSW control. (E) Summary of the effect of Gd^{3+} pretreatment on average flash intensity. There was no significant effect of Gd^{3+} pretreatment on average spontaneous flash intensity compared to the FSW control ($p = 0.9043$). Values in (B, C-E) represent the average ± SD of 8 replicates.

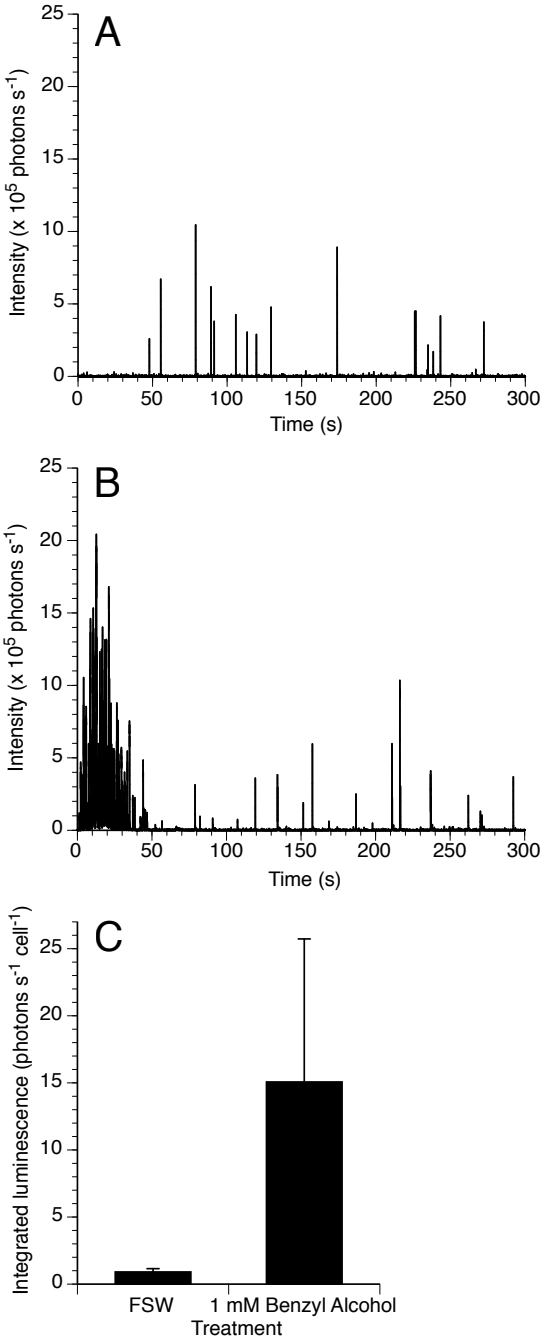

Figure 1.6. Stimulation of bioluminescence by benzyl alcohol. (A) Representative record of bioluminescence in ~2300 cells treated with the FSW control. (B) Similar representative record for cells treated with 1 mM benzyl alcohol. (C) Summary of integrated luminescence over 300 s in cells treated with FSW as the control or 1 mM benzyl alcohol. Stimulation of bioluminescence by benzyl alcohol was 16.6x higher and significantly different ($p < 0.0001$) than the FSW control.

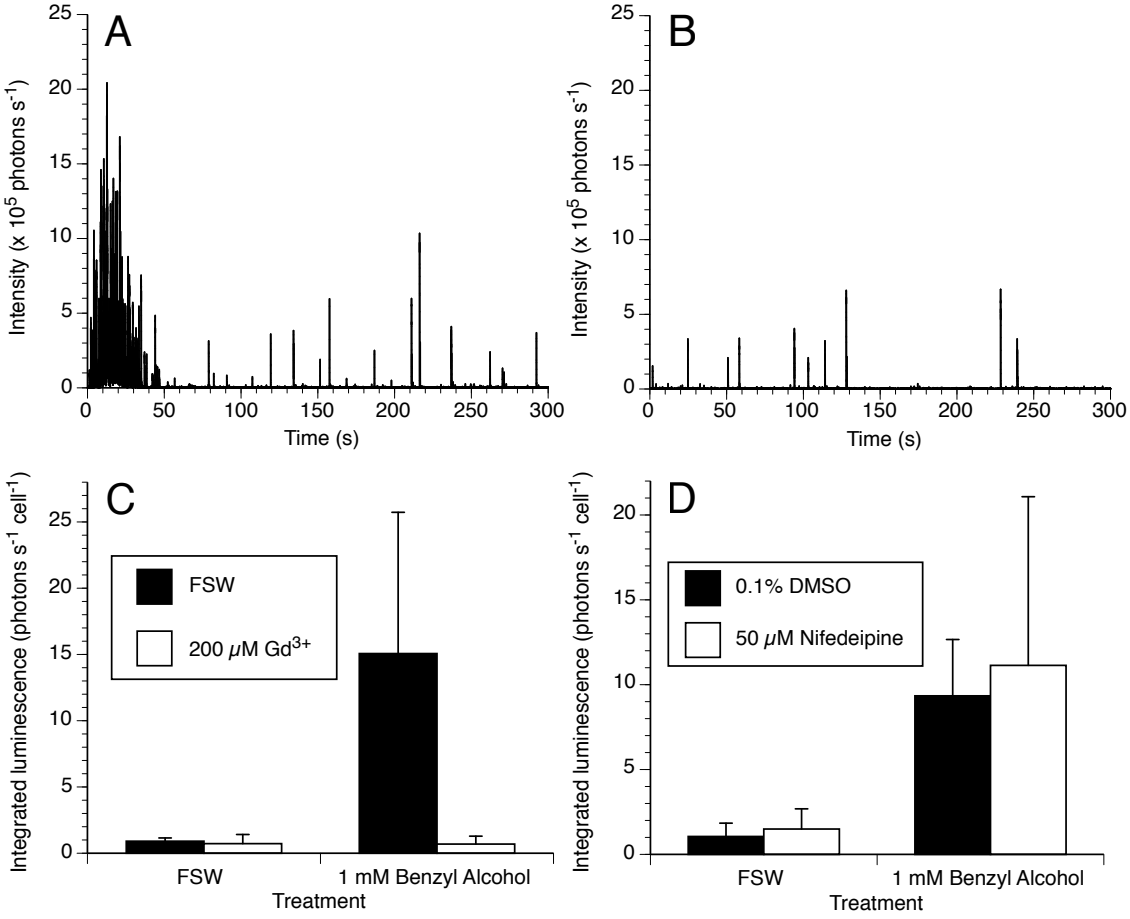

Figure 1.7. Effect of Gd^{3+} and nifedipine pretreatments on bioluminescence stimulation by benzyl alcohol. Representative records of 1 mM benzyl alcohol-stimulated bioluminescence in ~2300 cells pretreated with (A) FSW as the control or (B) 200 µM Gd^{3+}. (C) Summary of integrated luminescence in cells treated with FSW as the control or 1 mM benzyl alcohol and following pretreatment with FSW (closed bars) or 200 µM Gd^{3+} (open bars). The Gd^{3+} pretreatment inhibited benzyl alcohol stimulation by 95% compared to the FSW control pretreatment; this difference was significantly different ($p < 0.0001$). Values represent the average ± SD of 8 replicates. (D) Summary of integrated luminescence in cells treated with FSW as the control or 1 mM benzyl alcohol and then treated with 0.1% DMSO as the control (closed bars) or 50 µM nifedipine (open bars). There was no significant effect of nifedipine pretreatment on benzyl alcohol-induced bioluminescence ($p = 0.9044$). Values in (B-C) represent the average ± SD of 8 replicates.

Chapter II:

Mechanosensitive channels in dinoflagellates and their role in bioluminescence signaling and cell volume regulation

Summary

Dinoflagellate bioluminescence is a response to mechanical stress that triggers a cascade of signaling events that ultimately result in a physiological production of light. This pathway involves a stretch-sensitive mechanism based on non-specific pharmacological treatments and their effect on bioluminescence (chapter 1). Here, we seek to elucidate the identity of the stretch-activated proteins in the pathway, specifically the plasma membrane-bound ion channels that aid in the initial event of flow sensing. The two largest classes of eukaryotic ion channels thought to be involved in mechanotransduction include the Transient Receptor Potential (TRP) family of ion channels and the newly discovered Piezo mechanosensitive channel. In this study, we present the partial coding sequence of a TRP-like channel found from the expressed sequence tag (EST) database of *Symbiodinium sp*. and the partial coding sequence of a Piezo-like channel found in the EST database of *Karenia brevis*. In addition, two forms of bioluminescence – mechanically stimulated and spontaneous – were used as reporter systems to TRP channel-targeted pharmacological treatments in *L. polyedrum*. Treatment with 10 μM capsazepine, an inhibitor of the mammalian TRP villanoid receptor (TRPV)

family of channels, resulted in strong inhibition of both mechanically stimulated and spontaneous bioluminescence. Furthermore, treatment with 100 μM capsaicin, an activator of mammalian TRPV channels, very effectively stimulated bioluminescence. Hypoosmotic treatment, which caused osmotic swelling, was used to stimulate bioluminescence. Both production of light and osmotic swelling were attenuated or completely inhibited by both capsazepine and the non-specific stretch-activated ion channel antagonist Gadolinium III (Gd^{3+}), suggesting the role of TRP-like channels in both bioluminescence and osmotic sensing. These results are the first evidence of both TRP and Piezo mechanosensitive channels in dinoflagellates, and also strongly suggest the involvement of TRP channels in the bioluminescence signaling pathway and cell volume regulation of *L. polyedrum*.

Introduction

Mechanotransduction is a highly conserved component of cell signaling that allows for the conversion of mechanical stimuli into electrical or chemical signals and often involve ion channels that are directly gated by a mechanical stimulus. The ability for a single cell to directly sense mechanical stimuli is essential for any organism's ability to react to a rapidly changing environment. Mechanosensory channels have been characterized in processes such as hearing and pain reception in mammalian hair cells and peripheral sensory nerves (Bautista *et al*., 2006, Corey *et al*., 2004, Kwan *et al*., 2006), osmoregulation and hypertonic response in yeast (Denis & Cyert, 2002, Palmer *et al*., 2001), and response to shear stress in mammalian epithelial cells (Carattino *et al*., 2004).

A striking expression of mechanosensitive signal transduction pathway, however,

is seen in the dinoflagellate bioluminescent response to flow (Harrison, 1976, Rohr *et al*., 1998). As evident during red tides in breaking waves (Stokes *et al*., 2004) and in the wakes of ships or swimming animals (Rohr et al., 1998, Rohr *et al*., 2002) dinoflagellate bioluminescence is dependent on fluid shear stress (Latz *et al*., 1994, Latz & Rohr, 1999, Maldonado & Latz, 2007). However, its ecological relevance as a predator defense behavior is based on sensing the mechanical stress due to direct mechanical contact (Esaias & Curl, 1972, Fleisher & Case, 1995, Mensinger & Case, 1992).

In bioluminescent dinoflagellates, a Ca^{2+}-regulated pathway (von Dassow & Latz, 2002, von Dassow, 2003) governs the action potential that occurs across the vacuole membrane (Eckert, 1965, Widder & Case, 1981) and ultimately triggers the luciferin-oxidizing reaction that produces flashes of light (Fogel & Hastings, 1972, Wilson & Hastings, 1998). This entire process is initiated by the sensing of a mechanical stimulus at the plasma membrane (Mallipattu *et al*., 2002) that appears to activate a GTP-binding (G) protein (Chen *et al*., 2007).

In the previous chapter, it was suggested that the mechanically-stimulated bioluminescence signaling pathway is gated by a predominantly mechanosensitive, rather than voltage-sensitive mechanism. In the present study, we explore the possible identities of mechanosensitive ion channels in dinoflagellates and their role in the bioluminescence signaling pathway. There are many different types of mechanosensitive ion channels that have been characterized in eukaryotes (Arnadottir & Chalfie, 2010). This study highlights two specific classes of mechanosensitive channels that are predominant throughout the eukaryotic kingdom and display genetic conservation across species (Ramsey *et al*., 2006, Coste *et al*., 2010): transient receptor potential (TRP) ion channels and piezo

proteins.

TRP channels are a class of Ca^{2+} channels that have long been associated with mechanosensitive processes present in almost all eukaryotic organisms (Arnadottir & Chalfie, 2010). At least 19 putative TRP or TRP-like channels are known in the green alga *Chlamydomonas sp*. and a TRP-like channel is present in *Ostreococcus tauri* (Wheeler & Brownlee, 2008, Verret *et al*., 2010). Although the function of TRP channels in green algae is unclear, they are hypothesized to function similarly to mammalian TRP channels (Wheeler & Brownlee, 2008). Mammalian TRP channel proteins form six-transmembrane cation channels (Ramsey et al., 2006, Vannier *et al*., 1998) that can be divided into multiple subfamilies such as TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), etc (Ramsey et al., 2006). Mammalian TRPV and TRPC channels are known to play a role in osmoregulation (Gomis *et al*., 2008, Bourque *et al*., 2007, Sharif-Naeini *et al*., 2008, Sinke & Deen, 2011). In mouse neurons, TRPC channels have been found to associate with the $G_{q/11}$ family of G protein coupled receptors (GPCRs) (Okada *et al*., 1998, Schaefer *et al*., 2000), ultimately leading to the release of IP_3, which conduct the release of Ca^{2+} from intracellular stores (Schaefer et al., 2000). Based on the evidence for G protein activity and intracellular Ca^{2+}-regulation involved in bioluminescence (Chen et al., 2007, von Dassow, 2003), a similar pathway may function in dinoflagellates.

In contrast to the well-characterized TRP protein, piezo proteins are a novel class of ion channels that were recently discovered in mouse neuroblastoma cells and have homologs throughout the living kingdom (Coste et al., 2010). The exact role of piezo proteins in mechanosensing in vivo is yet unclear. However, overexpression in various mammalian cell lines resulted in large mechanosensitive cation currents (Coste et al.,

2010). Piezo proteins have yet to be pharmacologically characterized. However, many drugs that are known to inhibit TRP channels have been shown to inhibit piezo proteins as well (Coste et al., 2010, Bae *et al*., 2011), suggesting that the two may be pharmacologically indistinguishable.

TRP channels have been known to be involved in osmoregulation in mammals (Bourque et al., 2007, Sharif-Naeini et al., 2008, Sinke & Deen, 2011) allowing the use of the dinoflagellate bioluminescent response to hypoosmotic treatment as a potential reporter system for TRP channel activity. The mechanism by which dinoflagellates produce bioluminescence in response to hypoomostic conditions (Chen et al., 2007) is unknown. In other protists such as the ciliate *Paramecium sp*., osmoregulation is largely characterized by the sensing and regulation of $[Ca^{2+}]_i$ (Ladenburger *et al*., 2006, Verret et al., 2010). Because the dinoflagellate bioluminescence pathway is also characterized as Ca^{2+} dependent (von Dassow, 2003), we hypothesize that the mechanism of hypoosmotically-stimulated bioluminescence may involve the entry of extracellular Ca^{2+} into the cell upon sensing of osmotic pressure.

In the current study, partial sequences of mechanosensitive protein genes were identified from a dinoflagellate expressed sequence tag (EST) database. The two measureable forms of bioluminescence produced by *L. polyedrum*, mechanically stimulated (Maldonado & Latz, 2007) and spontaneous (Krasnow *et al*., 1980, Latz & Lee, 1995), were used as reporter systems for pharmacological treatments targeting mechanosensitive channels in the cell. Since TRP channels are also known to be involved in osmoregulation (Bourque et al., 2007, Sharif-Naeini et al., 2008, Sinke & Deen, 2011), hypoosmotic treatments were also used to induce cell swelling in *L. polyedrum*, which

was then inhibited with treatment with TRP channel antagonists.

Methods

Cell culture and preparation

Cultures of *Lingulodinium polyedrum* (Stein) Dodge strain CCMP1937 (formerly known as *Gonyaulax polyedra*) were grown in f/2 medium on a 12:12 h light-dark cycle in an environmental chamber maintained at 20.0±0.5°C. Cell concentrations were estimated on each day of testing by replicate counts of subsamples of known volume under a dissecting microscope. Experiments were performed on cultures within the exponential phase of growth with cell concentrations within the range of 1,000-8,000 cells ml^{-1}.

Subsamples of culture were prepared at the end of the light phase when cells are mechanically inexcitable (Biggley *et al*., 1969). Samples were placed in 7 ml glass scintillation vials. All samples were measured at 20.0±0.5°C, 4-7 h into the dark phase when maximal levels of bioluminescence are present (Biggley et al., 1969, Latz & Lee, 1995).

Experiments were typically set up using a 2 x 2 factorial design (chapter 1), with a 40 min pretreatment factor (inhibitor or control condition) and a treatment factor (activator or control condition). Each experiment consisted of four replicate samples for each combination of treatments (i.e., control pretreatment and control treatment, control pretreatment and activating treatment, inhibiting pretreatment and control treatment, and inhibiting pretreatment and activating treatment. Each experiment was replicated twice.

Media and chemicals

Filtered seawater (FSW) was prepared with GF/F filtered full-strength seawater

collected from Scripps Pier at University of California San Diego. MilliQ water was used to prepare 10% and 20% diluted seawater (90% and 80% FSW, respectively). Gadolinium (III) chloride (Gd^{3+}) and nifedipine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), capsazepine and capsaicin were purchased from Tocris Bioscience (Minneapolis, MN, USA), and dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Stock solutions of nifedipine, capsazepine, and capsaicin were prepared in DMSO and immediately diluted in FSW. Gadolinium (III) chloride solutions were prepared in FSW. All working solutions were made fresh daily. Concentrations represent the final concentration after addition of the chemical.

Bioluminescence measurements

Bioluminescence was measured in a 15 cm diameter light-integrating chamber (Labsphere Inc., North Sutton, NH, USA) using a photon-counting photomultiplier detector (Electron Tubes model P30232, ET Enterprises Ltd., Uxbridge, UK) fitted with a Uniblitz electronic shutter (Vincent Associates, Falmouth, MA, USA). A baffle blocked direct view of the sample vial by the detector, assuring an integrated light measurement due to highly reflective white inner surface of the chamber. In some cases the light signal was attenuated to 10% transmission with a 1.0 neutral density filter (Kodak Wratten #96, Eastman Kodak Co., Rochester, NY, USA) to avoid saturation of the detector. Light readings were acquired based on 10 ms integrations. Appropriate chemical and light pre-treatments were administered to each sample 40 min before testing. Chemical pretreatments, including controls, were manually administered slowly at 0.1 ml volumes to minimize prestimulation of bioluminescence. Spontaneous and injection-treated bioluminescence were recorded for 5 min. Stirring-induced bioluminescence was

recorded for 1 min. All experiments were carried out in a dim light environment to avoid photoinhibition of bioluminescence (Hamman & Seliger, 1982).

For measurements of spontaneous bioluminescence, samples were gently fixed in a holder and placed in the light-integrating chamber (Fig. 1.1 in chapter 1). Spontaneous flashes and glow were measured with the photomultiplier detector over 5 min without disturbance.

Chemical treatments, including capsaicin, 90% FSW, and the appropriate controls, were administered as described in chapter 1 at 0.1 ml volumes using a syringe pump (Harvard Apparatus, Holliston, MA, USA) at an infusion rate of 1 ml min^{-1} to minimize mechanical stimulation by the introduced liquid. Bioluminescence was then measured for 5 min. Bioluminescence mechanically stimulated by the addition of the fluid (usually the first 6 s) was not included in the final data analysis.

For mechanical stimulation of bioluminescence, samples were gently placed in a custom-designed holder that included a motorized stirrer as described in the previous chapter. Stirring-induced bioluminescence was then measured over 1 min. Stirring occurred at a constant rate throughout all experiments.

Assay for total luminescent capacity

Following bioluminescence measurements as previously described, residual light capacity of cells was assayed via cell acidification, This treatment causes the dissociation of luciferin from its binding protein and activation of luciferase, which is inactive at physiological pH, to oxidize any remaining luciferin. A Sirius luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) was used to simultaneously inject 250μl of 1 M acetic acid and measure light emission over 60 s, with the results expressed in

relative light units (RLU s^{-1} $cell^{-1}$). The sum of mechanical or chemical stimulation plus the luminometer acidification was the total stimulated luminescence (TSL), representing the luminescent capacity of the cells. TSL was used to assess the toxicity of pharmacological treatments. Unless otherwise stated, there was no significant effect of the treatments on TSL based on statistical analysis.

Cell-swelling measurements

Individual cell diameters were measured using the Multisizer™ 4 Coulter Counter (Beckman Coulter Inc., Indianapolis, IN, USA). Subsamples of culture were prepared at cell concentrations as previously mentioned, and prepared as well as measured during the light phase. Samples were placed in 20 ml polystyrene Accuvette cups. All samples were measured at 20.0±0.5°C.

Pretreatment solutions of Gd^{3+}, capsazepine, FSW, and DMSO were administered in 1 ml volumes 40 min prior to measurement. The volume of all pretreated samples was 10 ml. For the 100% and 90% FSW treatments, 1.1 ml of FSW or MilliQ water was added to the sample. For the 80% FSW treatment, 2.5 ml of MilliQ water was added to the sample. Samples were then immediately placed in the instrument and measured for cell size, based on equivalent spherical diameter based on the Coulter Principle. At least 3000 cells were measured from each sample.

Bioinformatics

Searches were conducted based on the entire dinoflagellate EST database with Basic Local Alignment Search Tool (BLAST) using candidate piezo and TRP channel proteins from various eukaryotic organisms (tBLASTn). These searches revealed ESTs from the dinoflagellate *Karenia brevis* that showed high sequence homology to piezo-like

mechanosensitive protein and from *Symbiodinium* sp. (Figure 2.2) that showed high sequence homology to mammalian TRP channels (Table 2.2).

Statistical analysis

Unless otherwise stated, all values are expressed as mean ± standard deviation. Statistical analyses were performed using JMP 9 software (SAS Institute Inc., Cary, NC, USA). Simple comparisons used Student's *t*-test. Analysis of variance for the 2 x 2 factorial experimental design was done with a mixed model using the REML (REsidual Maximum Likelihood) method, with the pretreatment and treatment conditions as fixed effects and experiment date as a random effect. Statistical significance was based on $P < 0.05$.

Results

Partial sequences of mechanosensitive channels in *Karenia brevis* and *Symbiodinium* sp.

A search using mammalian FAM38 proteins revealed one EST from the dinoflagellate *Karenia brevis* (Genbank FK849634). To confirm the identity of this EST as a piezo-like protein, the translated partial sequence was searched against the non-redundant (nr) protein database. The sequence from *Karenia brevis* showed similarities to piezo-like proteins from multiple different eukaryotic species (Table 2.1). Alignment with human and mouse piezo2 (Fig. 2.1) showed 20 out of 148 identical residues between the three sequences – a 14% identical match - and 82 out of 148 similar or identical residues between the three sequences – 55% similar identity match, suggesting the identity of this partial sequence from *Karenia brevis* may be a piezo-like protein.

A similar search using human TRP channel proteins also revealed one EST from the dinoflagellate *Symbiodinium* sp. (Genbank EH036498). The *Symbiodinium* sp. sequence

showed similarities to TRPC, TRP6, or TRP7 channels from multiple vertebrate organisms (Table 2.2). Alignment of this partial sequence with human TRPC6 and TRPC7 revealed conservation between the three sequences (Fig. 2.2). There were 62 identical residue matches between the three sequences, representing a 30% identical match, and 140 out of 204 similar or identical residue matches, representing a 67% similar or identical residue match between the three sequences. This high level of sequence resemblance suggests that the identity of the partial *Symbiodinium* sequence is a TRP-like channel.

These two partial sequences comprise the first evidence for the identity of mechanosensitive channels in dinoflagellates. Further pharmacological treatments were then used to explore the potential role mechanosensitive channels may play in bioluminescence signaling.

Capsazepine strongly inhibits stirring-induced and spontaneous bioluminescence

To target mechanosensitive ion channels in *L. polyedrum* and characterize their role in bioluminescence, pharmacological treatments were selected and optimized to maximize the inhibitory or activating effect (Table 2.3), while simultaneously minimizing cell toxicity. Pharmacological treatments were selected based on their effects on stretch-activated ion channels, mainly targeting TRP channels, as piezo channels have not yet been thoroughly pharmacologically characterized.

Capsazepine, an inhibitor of the mammalian TRP Villanoid (TRPV) family of ion channels (Bodding & Flockerzi, 2004, Liedtke *et al*., 2000, van Abel *et al*., 2005), was optimized to a concentration of 10 μM (data not shown) and used to target the predicted TRP-like channel in the bioluminescence signaling pathway of *L. polyedrum*.

Capsazepine strongly inhibited mechanically stimulated bioluminescence in *L. polyedrum* caused by stirring (Fig. 2.3A), suggesting the involvement of TRP-like channels in regulating bioluminescence. Stirring-stimulated integrated luminescence over 60 s the 0.15% DMSO control pretreatment was 206.0 ± 137.5 photons s^{-1} $cell^{-1}$, while that of the capsazsepine pretreatment was 87.2 ± 67.0 photons s^{-1} $cell^{-1}$; this 58% decrease represented a significant difference (*t*-test, $t_{14} = 2.18$, $p = 0.0470$)(Fig. 2.3B). TSL values for both the DMSO control pretreatment and capsazepine pretreatment were not significantly different (*t*-test, $t_{12} = 0.28$, $p = 0.7818$), indicating that capsazepine did not have a toxic effect on cells (data not shown).

Capsazepine also inhibited spontaneous bioluminescence (Fig. 2.3C, D). The integrated spontaneous luminescence over 300 s of the 0.15% DMSO control pretreatment was 0.8 ± 0.32 photons s^{-1} $cell^{-1}$, while that of the capsazepine pretreatment was 0.33 ± 0.06 photons s^{-1} $cell^{-1}$; this 58% decrease represented a significant difference (*t*-test, $t_{14} = 3.95$, $p = 0.0014$) (Fig. 2.3E). This inhibition was due to a decrease in spontaneous flash rate by capsazepine. The spontaneous flash rate of the DMSO control pretreatment was 13.8 ± 7.6 x 10^{-4} flashes $cell^{-1}$ min^{-1}, while that of the capsazepine pretreatment was 2.3 ± 1.5 x 10^{-4} flashes $cell^{-1}$ min^{-1}; this 84% decrease represented a significant decrease (*t*-test, $t_{14} = 4.20$, $p = 0.0009$) (Fig. 2.3F). There was no significant effect of capsazepine on spontaneous flash intensity (*t*-test, $t_{14} = 2.12$, $p = 0.0555$) (Fig. 2.3G).

The strong inhibition of capsazepine on spontaneous as well as mechanically stimulated bioluminescence suggests that TRP-like channels are present in *L. polyedrum* and that they play a role in the bioluminescence signaling pathway.

Capsaicin strongly activates bioluminescence and is inhibited by Gd^{3+}

Next, an activator of TRP channels was selected to further explore the potential involvement of TRP channels in the signaling pathway. Due to the strong effect of the antagonist, capsaicin, an agonist of mammalian TRPV channels (Bodding & Flockerzi, 2004, Liedtke et al., 2000, Smith *et al*., 2002, van Abel et al., 2005), was tested at an optimized concentration of 100 μM for treatment in *L. polyedrum* (data not shown). Capsaicin treatment caused immediate stimulation of bioluminescence (Fig. 2.4A,B). The integrated luminescence over 300 s for the 0.2% DMSO control treatment was 6.5 ± 11.6 photons s^{-1} $cell^{-1}$, while that for the 100 μm capsaicin treatment was 45.6 ± 39.3 photons s^{-1} $cell^{-1}$ (Fig2.4D); this 7x increase represented a significant difference (*t*-test, $t_{14} = 2.70$, $p = 0.0172$).

To further confirm the specificity of the capsaicin treatment, the activating treatment was administered to Gd^{3+}-pretreated cells. If capsaicin does target certain stretch-activated channels in *L. polyedrum* such as TRP-like channels, its activation of bioluminescence would be inhibited by pretreatment with Gd^{3+}, a non-specific antagonist of stretch-activated ion channels including TRP channels (Berrier *et al*., 1992, Hajduczok *et al*., 1994, Yang & Sachs, 1989). As predicted, pretreatment with 200 μM Gd^{3+} blocked the activation by 100 μM capsaicin (Fig. 2.4C,D). Overall the Gd^{3+} pretreatment inhibited capsaicin-stimulated bioluminescence by 78% compared to the FSW control pretreatment, with an integrated luminescence over 300 s of only 10.1 ± 13.3 photons s^{-1} $cell^{-1}$ for the Gd^{3+} pretreatment compared to 45.6 ± 39.3 photons s^{-1} $cell^{-1}$ for the FSW control (Fig. 2.5D); this represented a significant interaction (ANOVA, $F_{1,27} = 4.56$, $p = 0.0418$). TSL values for both the FSW control pretreatment and Gd^{3+} pretreatment were

not significantly different (ANOVA, $F_{1,26} = 2.80$, $p = 0.1061$), indicating that Gd^{3+} did not have a toxic effect on cells (data not shown).

The activation of bioluminescence by capsaicin further suggests the role of TRP-like channels involved in the signaling pathway. The inhibition of capsaicin-induced bioluminescence by Gd^{3+} further provides confidence in the specificity of the capsaicin treatment.

Hypoosmotic treatment activates bioluminescence and is inhibited by Gd^{3+} and capsazepine, but not nifedipine

TRP channels, specifically some members of the mammalian TRPV family channels, are activated by mechanical stimuli such as hypoosmotic and pressure-induced membrane stretching (Birder, 2007, Bossus *et al*., 2011, Fu *et al*., 2006, Gomis et al., 2008, Liedtke & Kim, 2005). To obtain further evidence of the presence of TRP-like channels in *L. polyedrum*, hypoosmotic treatment was used to stimulate bioluminescence and induce cell swelling (Chen et al., 2007).

Dilution to 90% FSW stimulated bioluminescence strongly stimulated bioluminescence (Fig. 2.5A,B). The integrated luminescence over 300 s for the FSW control treatment was $6.7 \pm 1.2 \times 10^{-1}$ photons s^{-1} $cell^{-1}$, while that for the 90% FSW treatment was $47.1 \pm 33.7 \times 10^{-1}$ photons s^{-1} $cell^{-1}$ (Fig. 2.5C); this 7x increase represented a highly significant difference (*t*-test, $t_{14} = 3.39$, $p = 0.0044$).

If the hypoosmotic treatment in *L. polyedrum* involves a TRP-like ion channel, then pretreatment with both Gd^{3+} and capsazepine should inhibit the stimulatory effect on bioluminescence of the 90% FSW treatment. As predicted, pretreatment with 200 μM Gd^{3+} inhibited hypoosmotic stimulation of bioluminescence by 89% compared to the

FSW control pretreatment, with an integrated luminescence over 300 s of 5.2 ± 1.7 x 10^{-1} photons s^{-1} $cell^{-1}$ for the Gd^{3+} pretreatment compared to 47.1 ± 3.7 x 10^{-1} photons s^{-1} $cell^{-1}$ for the FSW control (Fig. 2.5C); these results represented a significant interaction (ANOVA, $F_{1,27} = 10.88$, $p = 0.0027$). Gd^{3+} pretreatment also decreased bioluminescence of the FSW control treatment by 36%, representing a significant effect (ANOVA, $F_{1,27} = 13.69$, $p = 0.0010$) (Fig. 2.5C). There was no significant difference in TSL between the Gd^{3+} and control pretreatments (ANOVA, $F_{1,27} = 0.08$, $p = 0.7804$), indicating that Gd^{3+} did not have a toxic effect on cells (data not shown).

Similarly, pretreatment with 10 μM capsazepine inhibited the stimulatory effect on bioluminescence of the 90% FSW treatment by 72% compared to the 0.1% DMSO control pretreatment, with an integrated luminescence over 300 s of 5.0 ± 2.2 photons s^{-1} $cell^{-1}$ for the capsazepine pretreatment compared to 17.9 ± 5.8 photons s^{-1} $cell^{-1}$ for the DMSO control (Fig. 2.5D); there results represent a significant interaction (ANOVA, $F_{1,26} = 30.59$, $p < 0.0001$). There was no significant difference between the capsazepine and DMSO pretreatments due to an unexplained, high amount of variability in the capsazepine pretreatment samples (Fig. 2.5D). TSL for the capsazepine-pretreated cells was 10% higher and significantly different than TSL for the DMSO control pretreatment (ANOVA, $F_{1,26} = 24.26$, $p < 0.0001$), indicating that capsazepine-pretreated cells contained more residual bioluminescence as a result of inhibition and further indicating that capsazepine did not have a toxic effect on cells (data not shown).

The inhibition of hypoosmotic stimulation of bioluminescence by both Gd^{3+} and capsazepine is evidence for the presence of TRP-like channels in *L. polyedrum* and its involvement in osmoregulation and the bioluminescence signaling pathway.

To verify that a voltage-gated mechanism is not involved in the response to hypoosmotic treatment, cells were treated with nifedipine, an inhibitor of voltage-gated channels that inhibits mechanically stimulated and K^+-stimulated bioluminescence (von Dassow 2003; chapter 1). As expected, there was no significant interaction of 50 μM nifedipine pretreatment with hypoosmotic-stimulated bioluminescence (ANOVA, $F_{1,27} = 0.11$, $p = 0.7463$) (Fig. 2.5E); the integrated luminescence over 300 s after dilution to 90% FSW was 10.1 ± 3.0 photons s^{-1} $cell^{-1}$ for the nifedipine pretreatment and 9.6 ± 4.6 photons s^{-1} $cell^{-1}$ for the DMSO control pretreatment. TSL for the nifedipine and DMSO pretreatments were not significantly different (ANOVA, $F_{1,27} = 1.91$, $p = 0.1774$), indicating that nifedipine did not have a toxic effect on cells (data not shown).

Gd^{3+} and capsazepine inhibit hypoosmotic cell swelling

In *L. polyedrum*, cell size is dependent on the osmolarity of the medium; 90% FSW treatment causes a 2% increase in cell size, while 80% FSW treatment causes a 7% increase in cell size compared to controls in *L. polyedrum* strain HJ (Chen et al., 2007). This effect was replicated in the present study using strain CCMP1932 (Fig. 2.6A). The equivalent spherical diameter (ESD) in cells pretreated with FSW was 35.74 ± 0.03 μm (average ± standard error) for the control 100% FSW treatment, 36.25 ± 0.03 μm for the 90% FSW treatment representing a 1% increase, and 37.13 ± 0.04 μm for the 80% FSW treatment representing a 4% increase from the 100% FSW treatment (Fig. 2.6B). The differences in size were all significant (ANOVA, $F_5 = 215.75$, $p < 0.0001$).

If TRPV-like ion channels are involved in osmoregulation in dinoflagellates, treatment of cells with Gd^{3+} and capsazepine should attenuate the level of osmotic swelling. This effect was seen when Gd^{3+} pretreated cells were treated with 90% FSW,

but not 80% FSW (Fig. 2.6B). For the 100% FSW treatment, pretreatment with 200 μM Gd^{3+} resulted in a cell size of 35.07 ± 0.03 μm that represented a 1% decrease compared to an ESD of 35.26 ± 0.03 μm for the FSW pretreated control; while small, this change was still significantly different (ANOVA, $F_5 = 225.08$, $p = 0.0030$), suggesting that Gd^{3+} is impairing osmoregulation even for isosmotic conditions. For the 90% FSW treatment, Gd^{3+} pretreatment resulted in a cell size of 35.46 ± 0.04 μm that represented a 1% decrease compared to a cell size of 35.92 ± 0.03 μm for the FSW control pretreatment; this change was significantly different (ANOVA, $F_5 = 225.08$, $p < 0.0001$) (Fig. 2.7B). For the 80% FSW treatment, however, there was no significant difference in cell size between the Gd^{3+} pretreatment and FSW control pretreatment (ANOVA, $F_5 = 225.08$, $p = 0.3168$) (Fig. 2.7B). These results suggest Gd^{3+} pretreatment interferes with the regulation of cell size, but its action is limited, having no effect for the 80% FSW hypoosmotic treatment.

Pretreatment with capsazepine strongly inhibited cell swelling due to hypoosmotic treatment. For the 90% FSW treatment, pretreatment with 10 μM capsazepine pretreatment resulted in a cell size of 35.84 ± 0.03 μm that represented a 3% decrease compared to a cell size of 34.79 ± 0.03 μm for the DMSO control pretreatment (Fig. 2.7C), these results represented a significant interaction of capsazepine with the hypoosmotic treatment (ANOVA, $F_5 = 504.71$, $p < 0.0001$). Furthermore, capsazepine pretreatment also inhibited the effect of the 80% FSW treatment (Fig. 2.7C), resulting in a cell size of 35.00 ± 0.03 μm that represented a 5% decrease compared to a cell size of 36.72 ± 0.03 μm for the DMSO control pretreatment. These results also indicated a significant interaction (ANOVA, $F_5 = 504.71$, $p < 0.0001$). For the 100% FSW control

treatment, capsazepine pretreatment resulted in a <1% decrease in cell size of 34.80 ± 0.03 μm compared to the size of 34.97 ± 0.03 μm for the DMSO control pretreatment, nevertheless representing a significant difference (ANOVA, $F_5 = 504.71$, $p = 0.0173$) that suggests that capsazepine interferes with cell size regulation.

The inhibition by Gd^{3+} and capsazepine of hypoosmotic cell swelling provides evidence for a role of TRP-like channels in osmoregulation and cell size regulation in *L. polyedrum*, in addition to their involvement in the bioluminescence mechanosensitive signaling pathway.

Discussion

The results presented here provide evidence for the identity of mechanosensitive ion channels in dinoflagellates and their potential role in the bioluminescence signaling pathway and the regulation of cell volume. We present the first molecular evidence for the presence of mechanosensitive ion channels in dinoflagellates. Furthermore, the dramatic inhibition of both mechanically-stimulated and spontaneous bioluminescence by capsazepine and stimulation of bioluminescence by capsaicin suggest that either TRP-like or piezo-like mechanosensitive channels may place a role in mechanosensitive mechanism that regulates bioluminescence.

Mechanosensitive channels are present in both prokaryotes and eukaryotes (Arnadottir & Chalfie, 2010). Bacterial mechanosensitive channels such as MscL and MscS have been thoroughly characterized and found to be directly predominantly mechanically gated and weakly voltage gated (Bass *et al*., 2002, Chang *et al*., 1998, Vasquez *et al*., 2008). Similarly, TRP channels are weakly voltage-gated (Ramsey et al., 2006). It is controversial, however, whether or not TRP channels are directly gated by

mechanical stimuli; they may be a downstream component of a mechanosensitive pathway and not directly involved in mechanotransduction (Christensen & Corey, 2007, Gottlieb *et al*., 2008, Schnitzler *et al*., 2008, Sharif-Naeini et al., 2008). In mammalian cardiovascular cells, TRPC channels have been suggested to be downstream signaling molecules activated by a G protein coupled receptor (GPCR)-regulated pathway that senses mechanical stress (Sharif-Naeini *et al*., 2010, Wong & Yao, 2011). In cardiac myocytes, cells are proposed to respond to intraluminal pressure by means of a GPCR-gated mechanism. Gq proteins are couple to a mechanically-gated receptor, which can be activated by pressure, thereby causing the dissociation of Gq_{α} protein from its heterotrimeric conformation, which then activates $PLC_{ß}$, which cleaves PIP_2 into IP_3 and DAG. The DAG-sensitive TRPC channel then opens and allows extracellular Ca^{2+} to flow into the cell (Sharif-Naeini et al., 2010). This pathway proposed in mammalian endothelial cells shares many components with the dinoflagellate bioluminescence signaling pathway – mechanosensitivity, G-protein activation, possible TRP-like channel involvement, and intracellular Ca^{2+} regulation – suggesting the two pathways may function similarly.

Mechanisms for osmoregulation are not well characterized in dinoflagellates. Other protists including the ciliate *Paramecium sp*. and the green alga *Chlamydomonas reinhardtii* possess contractile vacuoles, osmoregulatory organelles that pump water in and out of the cell to regulate ion concentrations (Ishida *et al*., 1996, Stock *et al*., 2002). The molecular identities of the protein channels involved in osmoregulation in protists are largely unknown. There is, however, evidence for the presence and involvement of IP_3-gated channels in the regulation of Ca^{2+} at the contractile vacuoles (Ladenburger et

al., 2006, Wheeler & Brownlee, 2008). Despite their close taxonomic relationship, however, contractile vacuoles are not present in dinoflagellates, leaving room for speculation of alternate mechanisms of osmoregulation. TRP channels are involved in osmoregulation in higher eukaryotes (Bourque et al., 2007, Sharif-Naeini et al., 2008, Sinke & Deen, 2011). In mice, TRPV4 knockouts exhibit loss of osmotic and thermal sensitivity (Liedtke *et al*., 2003, Suzuki *et al*., 2003). A similar effect is also seen in the nematode *Caenorhabdatis elegans*, which contains osmosensitive channels called OSM-9, OCR-1, 2, 3, and 4 that are homologous to TRPV channels (Liedtke et al., 2003, Sokolchik *et al*., 2005). Nineteen putative TRP or TRP-like channels are known in the green algae *Chlamydomonas sp*. and a TRP channel homologue is present in *Ostreococcus tauri* (Wheeler & Brownlee, 2008, Verret et al., 2010) suggesting conservation of TRP-like channel function through eukaryotic evolution. In *L. polyedrum*, hypoomostic treatment stimulates bioluminescence and cell swelling (Chen et al., 2007, present study) by a mechanism that is not yet understood; presumably the osmotic treatment opens plasma membrane-bound ion channels that allow the influx of extracellular calcium into the cell resulting in an increase in $[Ca^{2+}]_{cyt}$ that triggers an action potential across the tonoplast membrane to activate the luminescent reaction. However, pretreatment with Gd^{3+} and capsazepine inhibited both bioluminescence and cell swelling. If the sensing of hypoosmotic conditions in *L. polyedrum* is regulated similarly by TRP-like channels, then the inhibition of cell swelling caused by hypoosmotic treatment provides further evidence for the presence of TRP-like channels in dinoflagellates.

Although the presence of both TRP and piezo channel genes in dinoflagellates does

not speak to the roles of these channels in regulating bioluminescence, it does demonstrate the dramatic level of conservation of signaling proteins through eukaryotic evolution and the essential role of mechanosensitive channels in cell function. The function of TRP channels is highly variable between subfamily and organism (Ramsey et al., 2006). However, most TRP channels can be characterized by sequence homology. The "TRP box" is a sequence of 6 amino acid residues that is conserved throughout most TRP channels, aside from TRPA and TRPP (Clapham, 2003, Ramsey et al., 2006). In human TRPV3 this sequence is IWRLQR (accession # Q8NET8), and in human TRPC channels it is EWKFAR. In the dinoflagellate *Symbiodinium*, it is VWKFSR (Figure 2.2) – a mere two-residue divergence from human TRPC channels. The structure and sequence of piezo mechanosensitive proteins have not been as thoroughly analyzed as TRP channels. However, piezo proteins have been evolutionarily conserved as well; 2 variants – piezo1 and piezo2 – have been found in vertebrates, while *Tetrahymena thermophilia* has 3 piezo homologs, and *Paramecium tetraurelia* has 6 homologs (Coste et al., 2010). Protein sequence conservation between such largely divergent kingdoms such as protists and animals suggest a highly essential role for mechanosensitive channels that has persisted throughout the evolution of eukaryotes.

The treatments used in this study are characterized for mammalian mechanosensitive channels, and have never before been used in a dinoflagellate. In addition, piezo and TRP proteins share similar sets of inhibitors, including Gd^{3+}, Ruthenium Red, and GsMTx-4 (Coste et al., 2010, Bae et al., 2011), suggesting that they are difficult to distinguish pharmacologically. Because of this, we cannot determine if the results of our TRP-specific pharmacological treatments were limited to only TRP-like

channels. It has also been difficult to sequence mechanosensitive genes from *L. polyedrum* due to lack of an availability of full-length dinoflagellate genomes. The dinoflagellate genome is uncommonly large, consisting of approximately 215,000 megabase pairs – almost 100x that of human cells – complicating efforts in full-genome sequencing (Dodge, 1985, Rizzo, 1991). Access to EST databases and soon-to-be-available dinoflagellate transcriptomes, however, will aid in future efforts to include obtaining full-length sequences of these mechanosensitive proteins, and studying their structure and function compared to vertebrate homologues. Nevertheless, the presence of mechanosensitive channels in dinoflagellates and their potential role in light production suggests an analogous relationship between the mechanosensitive processes in mammals – hearing, pain reception, osmoregulation, etc. – and dinoflagellate bioluminescence. If so, bioluminescent dinoflagellates present as an ideal potential model system of mechanosensitivity with simplified cell function and a convenient reporter system.

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Table 2.1. BLAST confirmation of identity of Piezo-type mechanosensitive channel from an expressed sequence tag from *Karenia brevis* (Genbank FK849634). Top 5 unique, annotated hits from BLASTx search using partial sequence from *K. brevis* are shown. Maximum identity indicates the percentage of residues in the partial *K. brevis* sequence that identically match the protein.

Genbank Number	Organism	Protein description	Identity (%)
XP_003339741	*Monodelphis domestica* (opossum)	Predicted Piezo2-like	36
XP_003626328	*Medicago truncatula* (barrel clover)	Fam28B	44
XP_002933721	*Xenopus tropicalis* (western clawed frog)	Fam28A-like	40
XP_696355	*Danio rerio* (zebrafish)	Piezo1	39
XP_001511171	*Ornithorhynchus anatinus* (platypus)	Piezo2-like	39

Table 2.2. BLASTx confirmation of identity of TRP-like channel partial nucleotide coding sequence in the dinoflagellate *Symbiodinium* sp. Top 5 unique, annotated hits from BLASTx search using an expressed sequence tag (Genbank EH036498) from *Symbiodinium* sp. are shown. Identity indicates the percentage of residues in the partial *Symbiodinium* sequence that identically match the protein.

Genbank Number	Organism	Protein description	Identity (%)
XP_002930504	*Ailuropoda melanoleuca* (giant panda)	Short TRP channel 6-like	36
AES08837	*Mustela putorius furo* (ferret)	TRP channel C6	35
XP_002935616	*Xenopus tropicalis* (western clawed frog)	Short TRP channel 6-like	35
XP_001367163	*Monodelphis domestica* (opossum)	Short TRP channel 7	34
XP_425214	*Gallus gallus* (chicken)	Short TRP channel 7	35

Table 2.3. Pharmacological treatments use in this study.

Chemical	Protein Target	Known/predicted effect on bioluminescence
Capsazepine	TRPV1, TRPM8 antagonist	Inhibits
Gadolinium III chloride (Gd^{3+})	Stretch-sensitive channel antagonist	Inhibits
Capsaicin	TRPV1 activator	Activates
Nifedipine	L-type Ca^{2+} channel antagonist	Inhibits

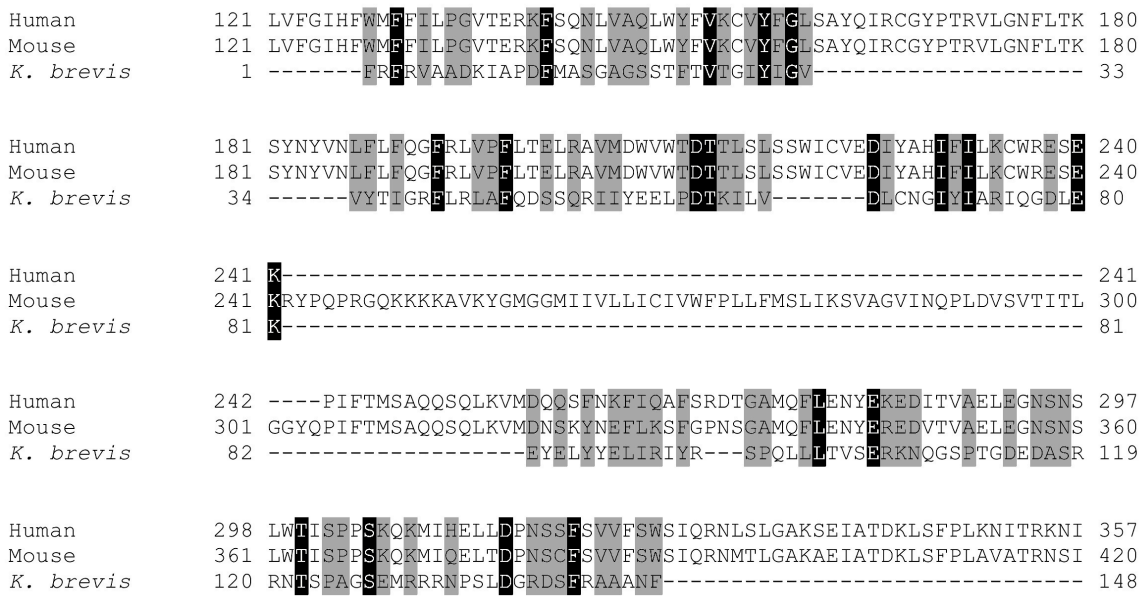

Figure 2.1. Piezo-like mechanosensitive channel partial protein sequence in *Karenia brevis*. Alignment of piezo-type mechanosensitive channel component 2 in human (Genbank BAB15641), *mouse* (*M. musculus,* Genbank AAI47607) and partial protein from *Karenia brevis* (Genbank FK849634*)*. Identical amino acids are shaded in black and similar amino acids are shaded in gray.

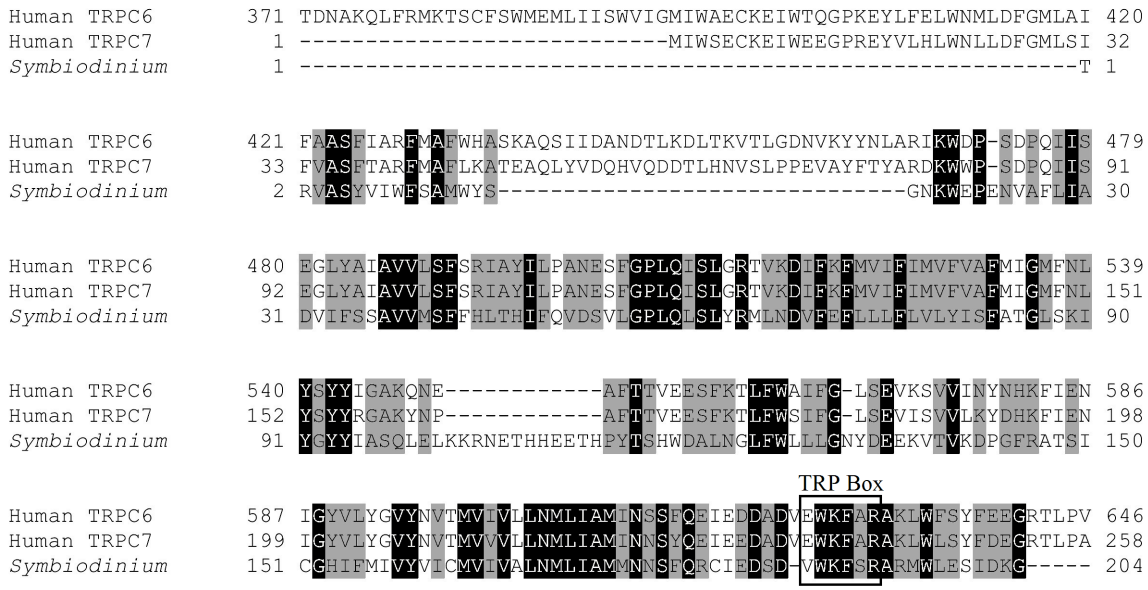

Figure 2.2. TRP-like channel partial protein sequence in the dinoflagellate *Symbiodinium* sp. Alignment of human TRPC6 (Genbank CAC01658), TRPC7 (Genbank EAW62188), and partial protein translated from *Symbiodinium* sp. EST (*Symbiodinium* sp., Genbank EH036498.1). Identical amino acids are shaded in black and similar amino acids are shaded in gray. Boxed area indicated conserved "TRP box" sequence.

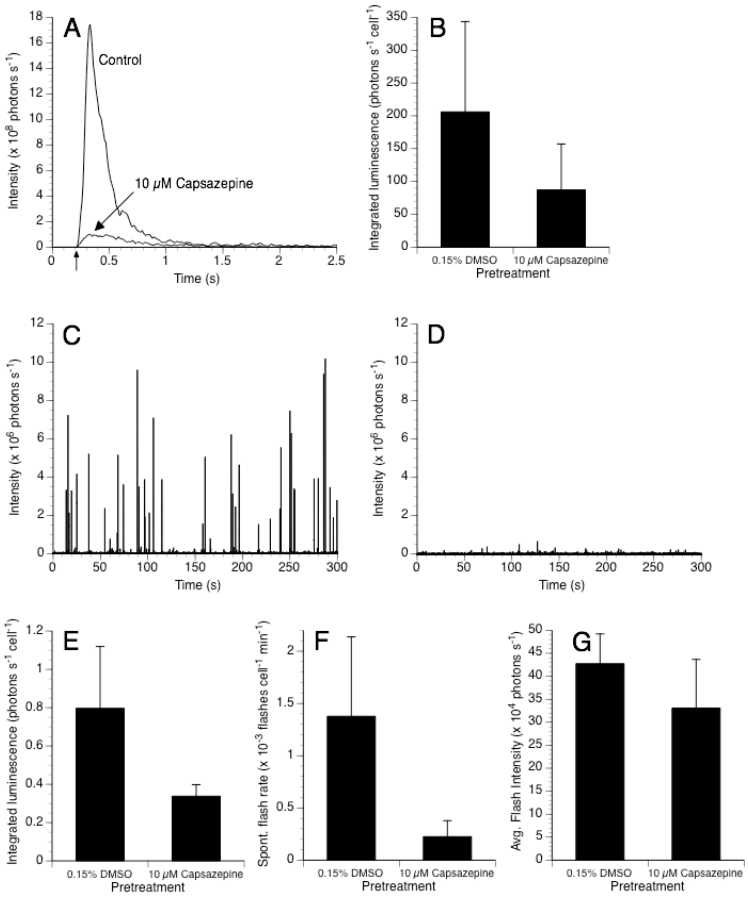

Figure 2.3. Effect of capsazepine pretreatment on mechanically-stimulated and spontaneous bioluminescence of *Lingulodinium polyedrum*. (A) Representative records of initial bioluminescence by ~3500 cells stimulated by stirring in cells pretreated with 0.1% DMSO as the control or 10 μM capsazepine. Arrow below the x-axis indicates the initiation of stirring. (B) Summary of the effect of capsazepine pretreatment on integrated stirring-induced luminescence over 60 s. Integrated bioluminescence for the capsazepine pretreatment was 58% lower and significantly different ($p = 0.0470$) than the DMSO control. (C) Representative record of spontaneous bioluminescence by ~3500 cells pretreated with the 0.1% DMSO control. (D) Similar record for cells pretreated with 10 μM capsazepine. (E) Summary of the effect of capsazepine on integrated spontaneous bioluminescence measured over 300 s. Integrated spontaneous bioluminescence for capsazepine pretreatment was 58% lower and significantly different ($p = 0.0014$) than the DMSO control. (F) Summary of the effect of capsazepine pretreatment on spontaneous flash rate. The spontaneous flash rate for capsazepine pretreatment was 84% lower and significantly different ($p = 0.0009$) than the DMSO control. (G) Summary of the effect of capsazepine pretreatment on average flash intensity. There was no significant effect of capsazepine pretreatment on average spontaneous flash intensity compared to the DMSO control ($p = 0.0555$). Values in (B, E-G) represent the average ± SD of 8 replicates.

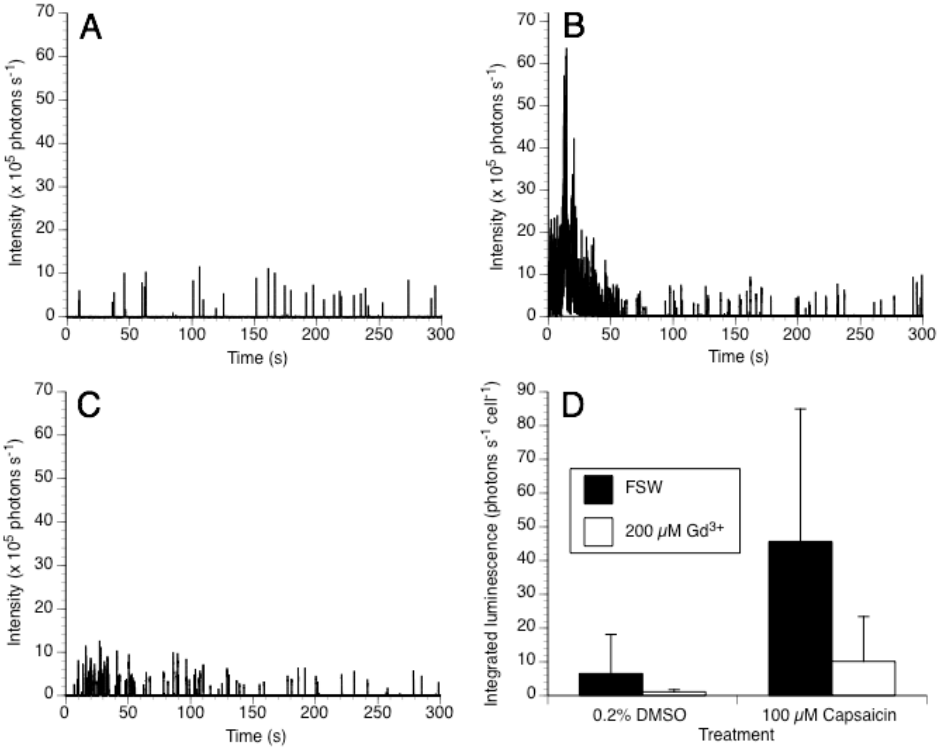

Figure 2.4. Stimulation of bioluminescence by capsaicin. Representative records of bioluminescence by ~4300 cells treated with (A) the 0.2% DMSO control or (B) 100 μM capsaicin.. (C) Similar record for cells pretreated with 200 μM Gd^{3+} and treated with 100 μM capsaicin. (D) Summary of integrated luminescence in cells pretreated with FSW as the control (closed bars) or 200 μM Gd^{3+} (open bars) and then treated with 0.2% DMSO or 100 μM capsaicin. The Gd^{3+} pretreatment inhibited capsaicin stimulation by 78% compared to the FSW control pretreatment; this difference was significantly different ($p = 0.0418$). Values represent the average ± SD of 8 replicates.

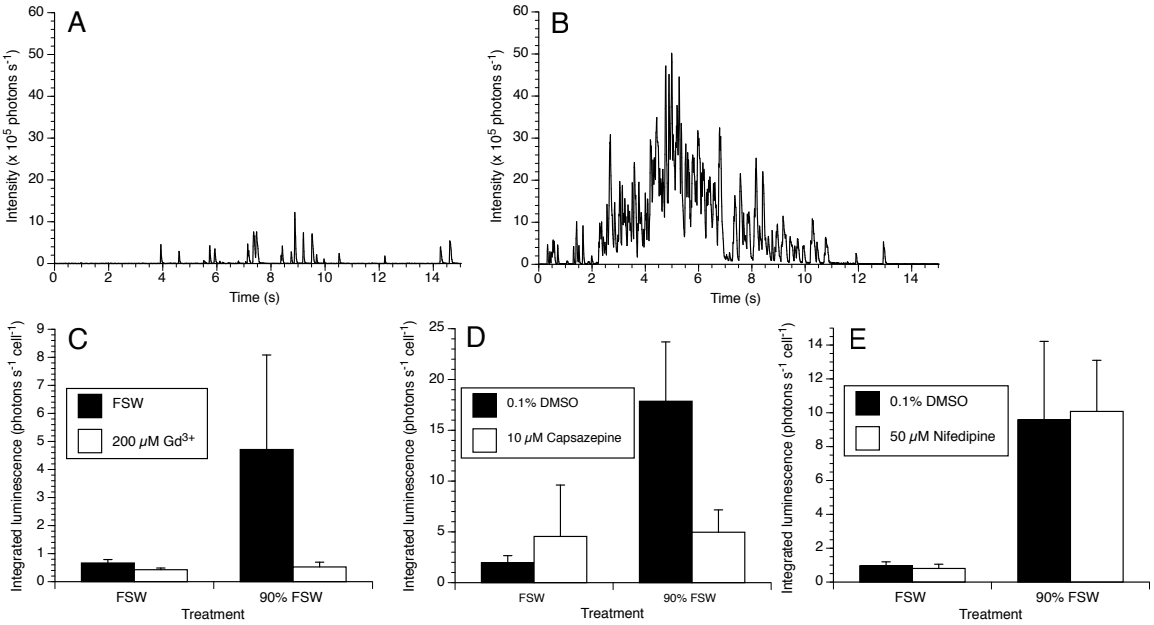

Figure 2.5. Effect of Gd^{3+}, capsazepine, and nifedipine pretreatments on hypo-osmotic bioluminescence stimulation. (A) Representative record of the first 15 s of bioluminescence released by ~3500 cells treated with the FSW control. (B) Similar record for cells treated with the hypo-osmotic treatment of 90% FSW. (C) Summary of integrated luminescence in cells pretreated with FSW as the control (closed bars) or 200 μM Gd^{3+} (open bars) and then treated with FSW or 90% FSW. The Gd^{3+} pretreatment inhibited hypo-osmotic stimulation by 89% compared to the FSW control pretreatment; this difference was significantly different ($p = 0.0027$). (D) Summary of integrated luminescence in cells pretreated with 0.1% DMSO as the control (closed bars) or 10 μM capsazepine (open bars) and then treated with FSW as the control or 90% FSW. The capsazepine pretreatment inhibited hypo-osmotic stimulation by 83% compared to the FSW control pretreatment; this difference was significantly different ($p = 0.0260$). (E) Summary of integrated luminescence in cells pretreated with 0.1% DMSO as the control (closed bars) or 50 μM nifedipine (open bars) and then treated with FSW as the control or 90% FSW. There was no significant effect of nifedipine pretreatment on hypo-osmotic induction of bioluminescence ($p = 0.7463$). Values in (C-E) represent the average ± SD of 8 replicates.

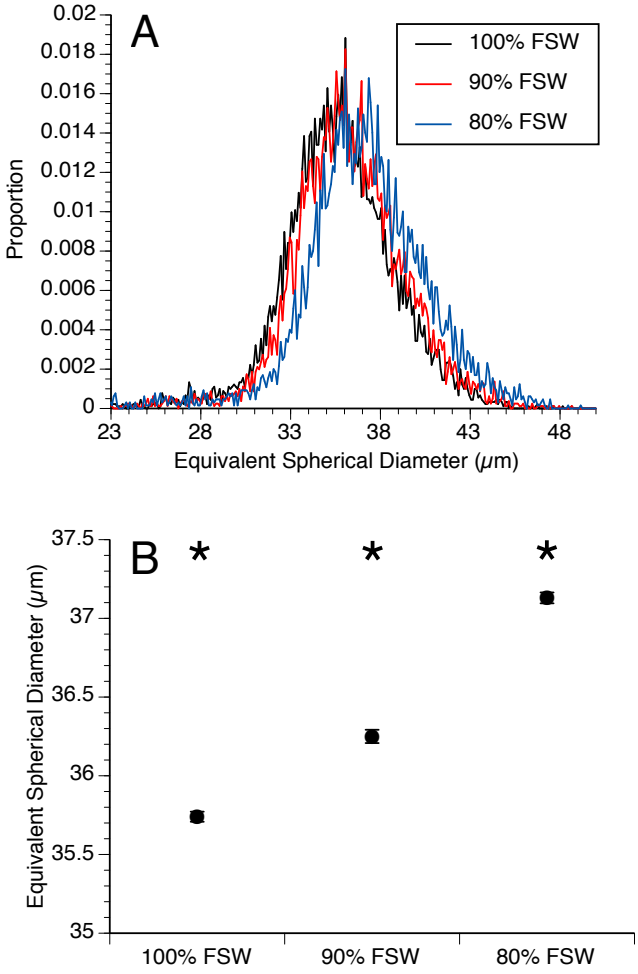

Figure 2.6. Effect of hypoosmotic treatment on cell diameter. (A) Distribution of cell sizes. Representative results of 3 replicates were totaled and are shown. Distribution represents ~9000 cells. (B) Summary of cell size in cells treated with 100%, 90% and 80% FSW. 90% FSW causes a 1% increase in average cell diameter compared to treatment with 100% FSW as the control; this difference was significantly different (ANOVA, $F_5 = 215.75$, $p < 0.0001$). 80% FSW causes a 4% increase in average cell diameter compared to 100% FSW controls; this difference was significantly different (ANOVA, $F_5 = 215.75$, $p < 0.0001$). Values represent the average ± standard error of ~9000 cells over 3 replicates.

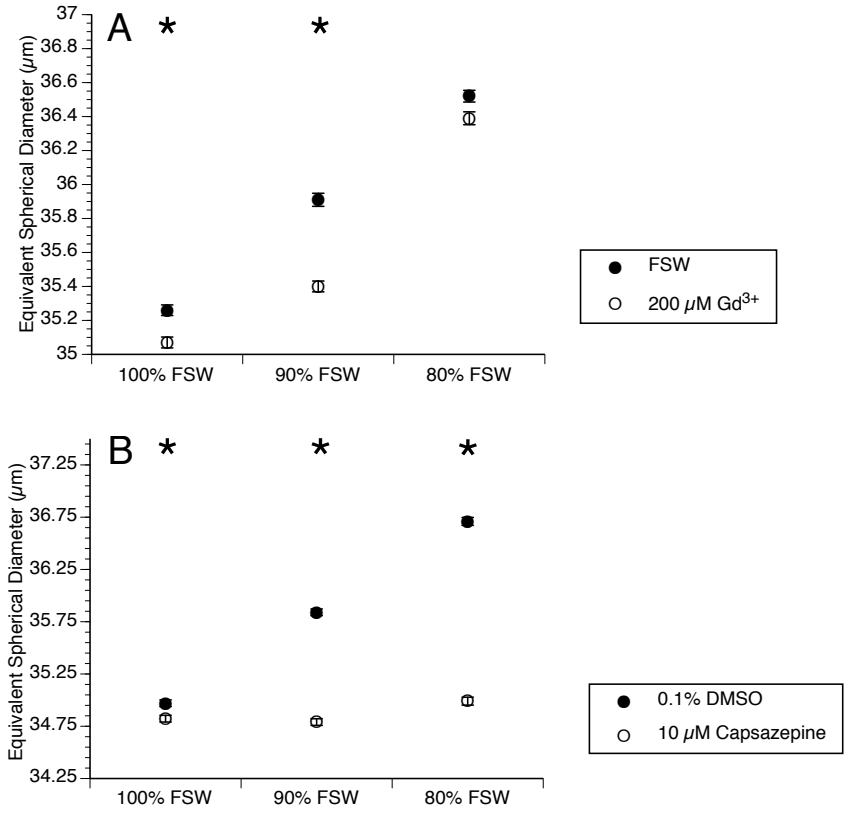

Figure 2.7. Effect of Gd^{3+} and capsazepine on hypoosmostic cell swelling of *Lingulodinium polyedrum*. (A) Summary of cell size for FSW or 200 μM Gd^{3+} pretreatments and then with 100%, 90%, or 80% FSW treatments. For the 100% and 90% FSW treatments, cell size for the Gd^{3+} pretreatment was 1% smaller than for the FSW pretreatment, representing a significant difference (ANOVA, $F_5 = 225.08$, $p < 0.0001$). For the 80% FSW treatment, there was no significant difference in cell size for the Gd^{3+} and FSW pretreatments (ANOVA, $F_5 = 225.08$, $p = 0.3168$). (B) Summary of cell size in cells for 0.1% DMSO (control) or 10 μM capsazepine pretreatments and then 100%, 90%, or 80% FSW treatments. For all treatments, capsazepine pretreatment resulted in a smaller cell size compared to the DMSO control (ANOVA, $F_5 = 504.71$, $p < 0.0001$). Asterisks (*) indicate a significant difference in comparison to the control. Values represent the average ± standard error (SE) of 3 replicates.

Chapter III:

Final conclusions about the dinoflagellate bioluminescence signaling pathway

Summary

In this concluding chapter, the future implications of the previously presented results are discussed. The knowledge gained from the work presented in this thesis is combined with previous studies to formulate a revised hypothesized signaling pathway for dinoflagellate bioluminescence. This pathway is proposed in the form of a flow chart. Additionally discussed are my personal thoughts on future research approaches that can be pursued to further shed light on the mechanisms of this signaling pathway.

A hypothesized partial signaling pathway for dinoflagellate bioluminescence

As previously discussed, there are many conserved aspects of the signaling events that govern mammalian endothelial cell flow-sensing and those that have been proposed to regulate dinoflagellate bioluminescence (chapter 2). Upon analysis of the conclusions from past and present pharmacological studies, and using knowledge from the much more well-characterized mechanosensing pathway in endothelial cells to "fill in the blanks", we present a model of the initial signaling pathway that regulates mechanically-stimulated dinoflagellate bioluminescence (Fig. 3.1). An initial event of mechanical stress may act on the plasma membrane to increase membrane fluidity (Mallipattu *et al*., 2002). This effect was mimicked by benzyl alcohol, which increases membrane fluidity and strongly stimulated bioluminescence (chapter 1). The presence of a stretch-sensitive

element in the pathway is based on pharmacological evidence: Gd^{3+}, an inhibitor of stretch-activated channels, inhibited benzyl alcohol-activated bioluminescence, while nifedipine, an inhibitor of voltage-gated (L-type) Ca^{2+} channels, did not (chapter 1). In mammalian endothelial cells, a G protein coupled receptor (GPCR) gating mechanism is proposed to regulate Ca^{2+}-mediated flow sensing (Sharif-Naeini *et al*., 2010, Wong & Yao, 2011). This GPCR is coupled to the Gq protein α subunit, which is directly activated by mechanical stress due to blood flow, thus being independent of ligand binding (Schnitzler *et al*., 2008, Yasuda *et al*., 2008, Zou *et al*., 2004). A Gq-like protein signaling pathway may mediate encystment in dinoflagellates (Tsim *et al*., 1996), and G proteins may be involved in the bioluminescence signaling pathway of *L. polyedrum* (Chen *et al*., 2007). Due to the role of intracellular Ca^{2+} in bioluminescence (von Dassow & Latz, 2002), Gq is hypothesized to be the identity of the G protein involved in the bioluminescence signaling pathway. Gq then activates the enzyme phospholipase C (PLC), which cleaves PIP_2, thereby releasing IP_3 and DAG. Following this cleavage, I hypothesize two methods of $[Ca^{2+}]_i$ increase: (1) release of Ca^{2+} from intracellular stores by an IP_3-gated mechanism, and (2) TRP-like channel activation by DAG which causes an influx of extracellular Ca^{2+} into the cytosol. In endothelial cells, TRPC6 is activated by DAG (Sharif-Naeini et al., 2010, Hofmann *et al*., 1999), which is consistent with this hypothesis. The presence of a TRP-like channel in dinoflagellates is based on the inhibition of bioluminescence by capsazepine, an inhibitor of mammalian TRPV channels, and activation of bioluminescence by capsaicin, an activator of mammalian TRPV channels (chapter 2). Together, the increase in $[IP_3]$ and the opening of TRP-like Ca^{2+} channels may act to increase $[Ca^{2+}]_i$ to the threshold needed to trigger the rest of the

pathway, which is believed to involve a Ca^{2+}-induced action potential across the vacuole membrane (Eckert, 1965, Widder & Case, 1981, Eckert & Sibaoka, 1968), the overall acidification of the scintillons, and the ultimate oxidation of luciferin, catalyzed by the pH-dependent luciferase (Fogel & Hastings, 1971, Wilson & Hastings, 1998).

Future advancements will be challenging, but not impossible

Due to the limited genetic tools currently available for dinoflagellates, including lack of a sequenced genome and lack of a transcript database, significant advancements in understanding the dinoflagellates' physiological processes are quite limited. The sensitive and delicate physiological properties of dinoflagellates make optimizing protocols very difficult and time-consuming. However, given these difficulties, there are certain areas that I believe show potential as future research approaches.

Given the identification of potential signaling proteins via pharmacological methods, the logical "next step" would be to genetically verify the presence of these proteins and express them in a different cell line for characterization. However, given the lack of genomic tools, any current efforts would be limited to primer design based on sequence conservation across related species. Thought not impossible, this process is very time-consuming and requires a bit of luck regarding the level of sequence conservation of your gene of interest. The availability of dinoflagellate transcriptome databases would highly aid in these efforts.

Although not the focus of the studies presented in this thesis, Ca^{2+} signaling plays a highly significant role in bioluminescence. Intracellular localization of this Ca^{2+} signal would be a remarkable contribution to understanding the dynamics of this pathway. This could be approached using fluorescent Ca^{2+} dyes, a protocol for which has been

previously designed in the dinoflagellate *Crypthecodinium cohnii* (Lam *et al*., 2005).

Dinoflagellate bioluminescence is a dramatic display of unicellular mechanosensng. For this reason, its use as a pharmacological reporter system is convenient. However, if dinoflagellates are ever to be considered as potential model systems for eukaryotic flow sensing, significant advancements in dinoflagellate genomics must be made. Definitive conclusions regarding properties of any signaling pathway cannot be made based on pharmacological data alone, and without the availability of molecular biochemistry techniques for a dinoflagellate system, definitive evidence will be difficult to obtain. However, with soon-to-be available dinoflagellate transcriptomes, the potential for advancement is great and will allow us to gain a better understanding of the level of conservation of mechanosensing through eukaryotic evolution, which may shed light on analogous processes in mammalian systems.

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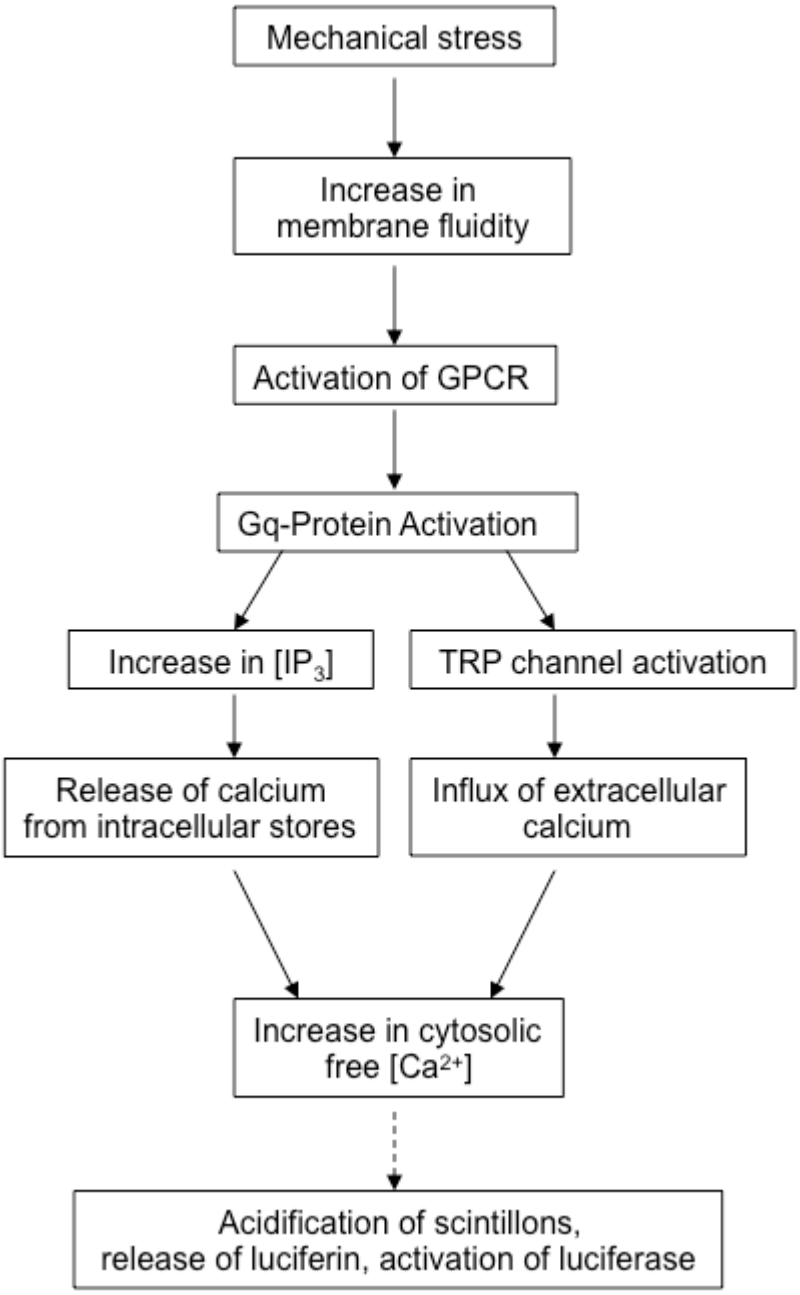

Figure 3.1. Flow chart indicating a modeled sequence of signaling events leading to the production of bioluminescence in dinoflagellates.